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(54) Title: TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHIA COLI

(57) Abstract

The present invention includes methods for generating neutralizing antitoxin directed against verotoxins. In particular, the antitoxin directed against these toxins is produced in avian species using soluble recombinant verotoxin proteins. This avian antitoxin is designed so as to be administrable in therapeutic amounts and may be in any form (i.e., as a solid or in aqueous solution). These antitoxins are useful in the treatment of humans and other animals intoxicated with at least one bacterial toxin, as well as for diagnostic assays to detect the presence of toxin in a sample.

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TREATMENT FOR VEROTOXIN-PRODUCING *ESCHERICHIA COLI*

FIELD OF THE INVENTION

The present invention relates to antitoxin therapy for humans and other animals. and diagnostic assays to detect toxins. Antitoxins which neutralize the pathologic effects of *Escherichia coli* toxins. such as verotoxin are provided.

BACKGROUND OF THE INVENTION

A. *Escherichia coli* as a Pathogenic Organism

Escherichia coli is the organism most commonly isolated in clinical microbiology laboratories. as it is usually present as normal flora in the intestines of humans and other animals. However. it is an important cause of intestinal. as well as extraintestinal infections. For example. in a 1984 survey of nosocomial infections in the United States. *E. coli* was associated with 30.7% of the urinary tract infections. 11.5% of the surgical wound infections. 6.4% of the lower respiratory tract infections. 10.5% of the primary bacteremia cases. 7.0% of the cutaneous infections. and 7.4% of the other infections (J.J. Farmer and M.T. Kelly. "Enterobacteriaceae." *in* Manual of Clinical Microbiology. Balows *et al.*(eds). American Society for Microbiology. [1991]. p. 365). Surveillance reports from England. Wales and Ireland for 1986 indicate that *E. coli* was responsible for 5.473 cases of bacteremia (including blood. bone marrow. spleen and heart specimens): of these. 568 were fatal. For spinal fluid specimens. there were 58 cases. with 10 fatalities (J.J. Farmer and M.T. Kelly. "Enterobacteriaceae." *in* Manual of Clinical Microbiology. Balows *et al.*(eds). American Society for Microbiology. [1991]. p. 366). There are no similar data for United States. as these are not reportable diseases in this country.

Studies in various countries have identified certain serotypes (based on both the O and H antigens) that are associated with the four major groups of *E. coli* recognized as enteric pathogens. Table 1 lists common serotypes included within these groups. The first group includes the classical enteropathogenic serotypes ("EPEC"): the next group includes those that produce heat-labile or heat-stable enterotoxins ("ETEC"): the third group includes the enteroinvasive strains ("EIEC") that mimic *Shigella* strains in their ability to invade and multiply within intestinal epithelial cells: and the fourth group includes strains and serotypes that cause hemorrhagic colitis or produce Shiga-like toxins (or verotoxins) ("VTEC" or "EHEC" [enterohemmorrhagic *E. coli*]).

Table 1.
Pathogenic *E. coli* Serotypes

Group	Associated Serotypes
Enterotoxigenic (ETEC)	O6:H16: O8:NM: O8:H9: O11:H27; O15:H11: O20:NM: O25:NM: O25:H42: O27:H7; O27:H20: O63:H12: O78:H11: O78:H12: O85:H7: O114:H21: O115:H21; O126:H9: O128ac:H7: O128ac:H12: O128ac:H21; O148:H28: O149:H4: O159:H4: O159:H20: O166:H27: and O167:H5
Enteropathogenic (EPEC)	O26:NM: O26:H11; O55:NM: O55:H6: O86:NM: O86:H2; O86:H34: O111ab:NM; O111ab:H2: O111ab:H12: O111ab:H21: O114:H2; O119:H6: O125ac:H21: O127:NM: O127:H6: O127:H9: O127:H21: O128ab:H2: O142:H6: and O158:H23
Enteroinvasive (EIEC)	O28ac:NM: O29:NM: O112ac:NM: O115:NM: O124:NM: O124:H7: O124:H30: O135:NM: O136:NM: O143:NM: O144:NM: O152:NM: O164:NM: and O167:NM
Verotoxin-Producing (VTEC))	O1:NM: O2:H5: O2:H7: O4:NM: O4:H10: O5:NM: O5:H16: O6:H1: O18:NM; O18:H7: O25:NM: O26:NM: O26:H11: O26:H32: O38:H21: O39:H4: O45:H2: O50:H7: O55:H7: O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM: O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10: O117:H4; O118:H12: O118:H30: O121:NM: O121:H19: O125:NM: O125:H8; O126:NM: O126:H8: O128:NM: O128:H2: O128:H8: O128:H12: O128:H25: O145:NM: O125:H25: O146:H21: O153:H25: O157:NM: O157:H7: O163:H19: O165:NM: O165:19: and O165:H25

B. Verotoxin Producing Strains of *E. coli*

Although all of these disease-associated serotypes cause potentially life-threatening disease. *E. coli* O157:H7 and other verotoxin-producing strains have recently gained widespread public attention in the United States due to their recently recognized association with two serious extraintestinal diseases. hemolytic uremic syndrome ("HUS") and thrombotic thrombocytopenic purpura ("TTP"). Worldwide. *E. coli* O157:H7 and other verotoxin-producing *E. coli* (VTEC) are an increasingly important human health problem. First identified as a cause of human illness in early 1982 following two outbreaks of food-related hemorrhagic colitis in Oregon and Michigan (M.A. Karmali. "Infection by Verocytotoxin-Producing *Escherichia coli*." Clin. Microbiol. Rev.. 2:15-38 [1989]: and L. W. Riley. *et al.* "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype." New Eng. J. Med..

308: 681-685 [1983]). the reported incidence of VTEC-associated disease has risen steadily. with outbreaks occurring in the U.S., Canada. and Europe.

With increased surveillance. *E. coli* O157:H7 has been recognized in other areas of the world including Mexico. China. Argentina. Belgium. and Thailand (N. V. Padhye and M. P. Doyle. "*Escherichia coli* O157:H7: Epidemiology, pathogenesis and methods for detection in food." J. Food. Prot., 55: 555-565 [1992]: and P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by *Escherichia coli* O157:H7. other enterohemorrhagic *E. coli*. and the associated hemolytic uremic syndrome." Epidemiol. Rev., 13: 60 [1991]).

The disease attracted national attention in the U.S. after a major outbreak in the Pacific Northwest that was associated with consumption of undercooked *E. coli* O157:H7-contaminated hamburgers. Over 700 hundred people fell ill (more than 170 were hospitalized) and four young children died (P. Recer. "Experts call for irradiation of meat to protect against food-borne bacteria." Associated Press. 7/12/94 [1994]). Several outbreaks since then have underscored the potential severity and multiple mechanisms for transmission of VTEC-associated diseases (M. Bielaszewská *et al.*, "Verotoxigenic (enterohaemorrhagic) *Escherichia coli* in infants and toddlers in Czechoslovakia." Infection 18: 352-356 [1990]: A. Caprioli *et al.*, "Hemolytic-uremic syndrome and Vero cytotoxin-producing *Escherichia coli* infection in Italy. "J. Infect. Dis., 166: 184-158 [1992]; A. Caprioli. *et al.*. "Community-wide Outbreak of Hemolytic-Uremic Syndrome Associated with Non-O157 Verocytotoxin-Producing *Escherichia coli*." J. Infect. Dis., 169: 208-211 [1994]; N. Cimolai. "Low frequency of high level Shiga-like toxin production in enteropathogenic *Escherichia coli* serogroups." Eur. J. Pediatr., 151: 147 [1992]; and R. Voelker., "Panel calls *E. coli* screening inadequate." *Escherichia coli* O157:H7--Panel sponsored by the American Gastroenterological Association Foundation in July 1994. Medical News & Perspectives. J. Amer. Med. Assoc., 272: 501 [1994]).

While O157:H7 is currently the predominant *E. coli* serotype associated with illness in North America. other serotypes (as shown in Table 1. and in particular O26:H11. O113:H21. O91:H21 and O111:NM) also produce verotoxins which appear to be important in the pathogenesis of gastrointestinal manifestations and the hemolytic uremic syndrome (P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by *Escherichia coli* O157:H7. other enterohemorrhagic *E. coli*. and the associated hemolytic uremic syndrome." Epidemiol. Rev., 13: 60 [1990]; M. M. Levine. *et al.*, "Antibodies to Shiga holotoxin and to two synthetic peptides of the B subunit in sera of patients with *Shigella dysenteriae* 1

dysentery." J. Clin. Microbiol., 30: 1636-1641 [1992]; and C. R. Dorn, *et al.*, "Properties of Vero cytotoxin producing *Escherichia coli* of human and animal origin belonging to serotypes other than O157:H7," Epidemiol. Infect., 103: 83-95 [1989]). Since organisms with these serotypes have been shown to cause illness in humans they may assume greater public health importance over time (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1990]).

Clinicians usually observe cases of hemolytic uremic syndrome ("HUS") clustered in a geographic region. However, small outbreaks are likely to be missed because many laboratories do not routinely screen stool specimens for *E. coli* O157:H7. Many cases related to non-commercial food preparation also probably go unrecognized. Nonetheless, *E. coli* O157:H7 is responsible for a large number of cases, as more than 20,000 cases of *E. coli* O157:H7 infection are reported annually in the U.S., with 400–500 deaths from HUS. However, these estimates were compiled when only 11 states mandated reporting of *E. coli* O157:H7. Twenty-nine states have recently made *E. coli* O157:H7 infection a reportable disease (R. Voelker, "Panel calls *E. coli* screening inadequate: *Escherichia coli* O157:H7; panel sponsored by the American Gastroenterological Association Foundation in July 1994, Medical News & Perspectives," J. Amer. Med. Assoc., 272: 501 [1994]). Indeed, the Centers for Disease Control recently added *E. coli* O157:H7 to their list of reportable diseases ("Public Health Threats," Science 267:1427 [1995]).

C. Nature of Verotoxin-Induced Disease

Risk factors for HUS progression following infection with *E. coli* O157:H7 include age (very young or elderly), bloody diarrhea, leukocytosis, fever, large amounts of ingested pathogen, previous gastrectomy, and the use of antimicrobial agents (in particular, trimethoprim-sulfamethoxazole)(A. A. Harris *et al.*, "Results of a screening method used in a 12 month stool survey for *Escherichia coli* O157:H7," J. Infect. Dis., 152: 775-777 [1985]; and M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 [1989]).

As indicated above, *E. coli* O157:H7 is associated with significant morbidity and mortality. The spectrum of illness associated with *E. coli* O157:H7 infection includes asymptomatic infection, mild uncomplicated diarrhea, hemorrhagic colitis, HUS, and TTP". Hemorrhagic colitis (or "ischemic colitis") is a distinct clinical syndrome characterized by

sudden onset of abdominal cramps—likened to the pain associated with labor or appendicitis—followed within 24 hours by watery diarrhea. One to two days later, the diarrhea turns grossly bloody in approximately 90% of patients and has been described as "all blood and no stool" (C. H. Pai *et al.*, "Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7," Ann. Intern. Med., 101: 738-742 [1984]; and R. S. Remis *et al.*, "Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7," Ann. Intern. Med., 101: 738-742 [1984]). Vomiting may occur, but there is little or no fever. The time from ingestion to first loose stool ranges from 3–9 days (with a mean of 4 days) L. W. Riley *et al.*, "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," New Eng. J. Med., 308: 681-685 [1983]; and D. Pudden *et al.*, "Hemorrhagic colitis in a nursing home," Ontario Can. Dis. Weekly Rpt., 11: 169-170 [1985]), and the duration of illness ranges generally from 2–9 days (with a mean of 4 days).

HUS is a life-threatening blood disorder that appears within 3–7 days following onset of diarrhea in 10–15% of patients. Those younger than 10 years and the elderly are at particular risk. Symptoms include renal glomerular damage, hemolytic anemia (rupturing of erythrocytes as they pass through damaged renal glomeruli), thrombocytopenia and acute kidney failure. Approximately 15% of patients with HUS die or suffer chronic renal failure. Indeed, HUS is a leading cause of renal failure in childhood (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 [1989]). Currently, blood transfusion and dialysis are the only therapies for HUS.

TTP shares similar histopathologic findings with HUS, but usually results in multiorgan microvascular thrombosis. Neurological signs and fever are more prominent in TTP, compared with HUS. Generally occurring in adults, TTP is characterized by microangiopathic hemolytic anemia, profound thrombocytopenia, fluctuating neurologic signs, fever and mild azotemia (H. C. Kwaan, "Clinicopathological features of thrombotic thrombocytopenic purpura," Semin. Hematol., 24: 71-81 [1987]; and S. J. Machin, "Clinical annotation: Thrombotic thrombocytopenic purpura," Br. J. Hematol., 56: 191-197 [1984]). Patients often die from microthrombi in the brain. In one review of 271 cases, a rapidly progressive course was noted, with 75% of patients dying within 90 days (E.L. Amorosi and J.E. Ultmann, "Thrombotic thrombocytopenic purpura: Report of 16 cases and review of the literature," Med., 45:139-159 (1966).

Other diseases associated with *E. coli* O157:H7 infection include hemorrhagic cystitis and balantitis (W. R. Grandsen *et al.*, "Hemorrhagic cystitis and balantitis associated with

verotoxin-producing *Escherichia coli* O157:H7." Lancet ii: 150 [1985]). convulsions. sepsis with other organisms and anemia (P. C. Rowe *et al.*, "Hemolytic anemia after childhood *Escherichia coli* O157:H7 infection: Are females at increased risk?" Epidemiol. Infect.. 106: 523-530 [1991]).

D. Mechanism of Pathogenesis

Verotoxins are strongly linked to *E. coli* O157:H7 pathogenesis. All clinical isolates of *E. coli* O157:H7 have been shown to produce one or both verotoxins (VT1 and VT2) (C. A. Bopp *et al.*, "Unusual Verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis." J. Clin. Microbiol.. 25: 1486-1489 [1987]). Both of these toxins are cytotoxic to Vero (African green monkey kidney) and HeLa cells. and cause paralysis and death in mice (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis." *Lancet* ii: 573 [1983]). These toxins are sometimes referred to in the literature as Shiga-like toxins I and II (SLT-I and SLT-II. respectively). due to their similarities with the toxins produced by *Shigella*. Indeed. much of our understanding of *E. coli* VTs is based on information accumulated on Shiga toxins. Shiga toxin. first described in 1903. has been recognized as one of the most potent bacterial toxins for eukaryotic cells (reviewed by M.A. Karmali. "Infection by Verocytotoxin-producing *Escherichia coli*." Clin. Microbiol. Rev.. 2: 15-38 [1989]). Hereinafter. the VT convention will be used: thus. VT1 and VT2 correspond to SLT-I and SLT-II. respectively.

While the pathogenic mechanism of *E. coli* O157:H7 infection is incompletely understood. it is believed that ingested organisms adhere to and colonize the intestinal mucosa. where toxins are released which cause endothelial cell damage and bloody diarrhea. It is also postulated that hemorrhagic colitis progresses to HUS when verotoxins enter the bloodstream. damaging the endothelial cells of the microvasculature and triggering a cascade of events resulting in thrombus deposition in small vessels. These microthrombi occlude the microcapillaries of the kidneys (particularly in the glomeruli) and other organs. resulting in their failure (J. J. Byrnes and J. L. Moake. "TTP and HUS syndrome: Evolving concepts of pathogenesis and therapy." Clin. Hematol.. 15: 413-442 [1986]: and T. G. Cleary. "Cytotoxin-producing *Escherichia coli* and the hemolytic uremic syndrome." Pediatr. Clin. North Am.. 35: 485-501 [1988]). Verotoxins entering the bloodstream may also result in direct kidney cytotoxicity.

VT1 is immunologically and structurally indistinguishable from Shiga toxin produced by *Shigella dysenteriae* (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis." *Lancet* ii: 573 [1983]). VT1 and VT2 holotoxins each consist of one A and five B subunits (A. Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies," Infect. Immun., 57: 3888-3893 [1989]; and A. Donohue-Rolfe *et al.*, "Simplified high yield purification of *Shigella* toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies," J. Exp. Med., 160: 1767-1781 [1984]). The toxic A subunit is enzymatically active, while the B subunit binds the holotoxin to the receptor on the target eukaryotic cell.

Crystal structure analysis of Shiga holotoxin and VT1 B subunit pentamers have shown that the holotoxin assembles with the C-terminal end of the A subunit associating with, and inserting within, a pentamer of B chains (P. E. Stein *et al.*, "Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*," Nature 355: 748-750 [1992]; and M.E. Fraser *et al.*, "Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution," Struct. Biol., 1:59-64 [1994]). This conformation is consistent with the observation that a C-terminally truncated A1 subunit of VT1 is toxic (in a ribosomal inhibition assay), but cannot associate with B subunit pentamers (P. R. Austin *et al.* "Evidence that the A_2 fragment of Shiga-like toxin type I is required for holotoxin integrity," Infect. Immun., 62: 1768 [1994]).

The Verotoxin A Subunit. Examination of the crystal structure of Shiga holotoxin indicates that the N-terminus of its A subunit is both surface-exposed and functionally important. Removal of amino acid interval 3–18 of the A subunit completely abolished toxicity (L. P. Perera *et al.*, "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II," Infect. Immun., 59: 829-835 [1991]) while removal of interval 25–44 retained toxicity but abolished its association with B subunit pentamers (J. E. Haddad *et al.*, "Minimum domain of the Shiga toxin A subunit required for enzymatic activity," J. Bacteriol., 175: 4970-4978 [1993]). Deletion of the first 13 residues of the homologous ricin A subunit also abolished toxicity, while deletion of the first 9 residues did not (M. J. May, *et al.*, "Ribosome inactivation by ricin A chain: A sensitive method to assess the activity of wild-type and mutant polypeptides. " EMBO J., 8: 301-308 [1989]).

The Verotoxin B Subunit. Studies of Shiga toxin B subunit suggest that neutralizing epitopes may also be present at both the N- and C-terminal regions of VT1 and VT2 B

subunits. Polyclonal antibodies raised against peptides from these regions (residues 5–18. 13–26. 7–26. 54–67 and 57–67) show partial neutralization of Shiga toxin (I. Harari and R. Arnon. "Carboxy-terminal peptides from the B subunit of Shiga toxin induce a local and parenteral protective effect." Mol. Immunol.. 27: 613-621 [1990]: and I. Harari *et al.*. "Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity." Infect. Immun., 56: 1618-1624 [1988]). Deletion of the last five amino acids of Shiga toxin B (M. P. Jackson *et al.*. "Functional Analysis of the Shiga toxin and Shiga-like toxin Type II variant binding subunits by using site-directed mutagenesis." J. Bacteriol.. 172: 653-658 [1990]). or four amino acids of VT2 B (L. P. Perera *et al.*. "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II." Infect. Immun.. 59: 829-835 [1991]). eliminate toxin activity, while deletion of the last two amino acids of VT2 B subunit reduced cytotoxicity. In contrast. the addition of an 18 or 21 amino acid extension to the native C-terminus of the VT2 B subunit was presumably conformationally correct. as these proteins assembled cytotoxic holotoxin.

Various approaches to express recombinant verotoxins have included individual or coordinate expression of A and B subunits from high-copy number plasmids and expression with fusion partners (J. E. Haddad *et al.*. "Minimum domain of the Shiga toxin A subunit required for enzymatic activity." J. Bacteriol., 175: 4970-4978 : J. E. Haddad. and M. P. Jackson. "Identification of the Shiga toxin A-subunit residues required for holotoxin assembly." J. Bacteriol.. 175: 7652-7657 [1993]: M. P. Jackson *et al.*. "Mutational analysis of the Shiga toxin and Shiga-like toxin II enzymatic subunits." J. Bacteriol.. 172: 3346-3350 [1990]: C. J. Hovde *et al.*. "Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I." Proc. Natl. Acad. Sci., 85: 2568-2572 [1988]: R. L. Deresiewicz *et al.*. "The role of tyrosine-114 in the enzymatic activity of the Shiga-like toxin I A-chain." Mol. Gen. Genet.. 241: 467-473 [1993]: T. M. Zollman *et al.*. "Purification of Recombinant Shiga-like Toxin Type I A_1 Fragment from *Escherichia coli*." Protein Express.Purific.. 5: 291-295 [1994]: K. Ramotar. *et al.*. "Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron." Biochem J.. 272: 805-811 [1990]: S. B. Calderwood *et al.*. "A system for production and rapid purification of large amounts of the Shiga toxin/Shiga-like toxin I B subunit." Infect. Immun.. 58: 2977-2982 [1990]: D. W. K. Acheson. *et al.*. "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]: M. P. Jackson *et al.*. "Nucleotide sequence analysis and

comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," FEMS Microbiol. Lett., 44: 109-114 [1987]; J. W. Newland *et al.*, "Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II." Infect. Immun. 55: 2675-2680 [1987]; and F. Gunzer and H. Karch. "Expression of A and B subunits of Shiga-like toxin II as fusions with glutathione *S*-transferase and their potential for use in seroepidemiology.". J. Clin. Microbiol., 31: 2604-2610 [1993]; and D.W. Acheson *et al.*, "Expression and purification of Shiga-like toxin II B subunits." Inf. Immun., 63:301-308 [1995]). In one case, bench top fermentation techniques yielded 22 mg/liter of soluble recombinant protein (D. W. K. Acheson, *et al.*, "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]). However, there have been no systematic approaches to identifying the appropriate spectrum of VT antigens, preserving immunogen and immunoabsorbant antigenicity and scaling-up.

The receptor for VT1 and VT2 is a globotriaosyl ceramide containing a galactose α-(1-4)- galactose-β-(1-4) glucose ceramide (Gb3) (C. A. Lingwood *et al.*, "Glycolipid binding of natural and recombinant *Escherichia coli* produced verotoxin *in vitro*." J. Biol. Chem., 262: 1779-1785 [1987]; and T. Wadell *et al.*, "Globotriaosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2." Biochem. Biophys. Res. Commun., 152: 674-679 [1987]). Gb3 is abundant in the cortex of the human kidney and is present in primary human endothelial cell cultures. Hence, the identification of Gb3 as the functional receptor for VT1 and VT2 is consistent with their role in HUS pathogenesis, in which endothelial cells of the renal vasculature are the principal site of damage. Therefore, toxin-mediated pathogenesis may follow a sequence of B subunit binding to Gb3 receptors on kidney cells, toxin internalization, enzymatic reduction of the A subunit to an A1 fragment, binding of the A1 subunit to the 60S ribosomal subunit, inhibition of protein synthesis and cell death (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]).

The role of verotoxins in the pathogenesis of *E. coli* O157:H7 infections has been further studied in animal models. Infection or toxin challenge of laboratory animals do not produce all the pathologies and symptoms of hemorrhagic colitis, HUS, and TTP which occur in humans. Glomerular damage is noticeably absent. Nonetheless, experiments using animal models implicate verotoxins as the direct cause of hemorrhagic colitis, microvascular damage leading to the failure of kidneys and other organs and CNS neuropathies.

For example, Barrett, *et al.* delivered VT2 into the peritoneal cavity of rabbits using mini-osmotic pumps (J. J. Barrett *et al.*, "Continuous peritoneal infusion of shiga-like toxin II (SLTII) as a model for SLT II-induced diseases," J. Infect. Dis., 159: 774-777 [1989]). In three days, most animals receiving the toxin developed diarrhea, with intestinal lesions resembling those seen in humans with hemorrhagic colitis. Although there was some evidence of renal dysfunction, none of the rabbits developed HUS. Beery, *et al.* showed that VT2, when administered intraperitoneally or intravenously to adult mice, produces lesions of the kidneys and colon (J. T. Beery *et al.*, "Cytotoxic activity of *Escherichia coli* O157:H7 culture filtrate on the mouse colon and kidney," Curr. Microbiol., 11: 335-342 [1984]). Histologic lesions in the kidney included accumulation of numerous exfoliated collecting tubules and marked intracellular vacuolation of proximal convoluted tubular cells.

Sjögren *et. al.* studied the pathogenesis of an entero-adherent strain of *E. coli* (RDEC-1) lysogenized with a VT1-containing bacteriophage (VT1-producing RDEC-1) (R. Sjögren *et al.*, "Role of Shiga-like toxin I in bacterial enteritis: comparison between isogenic *Escherichia coli* strains induced in rabbits," Gastroenterol., 106: 306-317 [1994]). In this study, rabbits were challenged with RDEC-1 or VT1-producing RDEC-1 and studied for onset of disease. The VT1-producing variant induced a severe, non-invasive, entero-adherent infection in rabbits which was characterized by serious histological lesions with vascular changes, edema and severe epithelial inflammation. Importantly, vascular changes consistent with endothelial damage were seen in infected animals that was similar to intestinal microvascular changes in humans with *E. coli* O157:H7 infection. Based on these observations, they concluded that VT1 is an important virulence factor in enterohemorrhagic *E. coli* O157:H7 infection.

Fuji *et. al.* described a model in which mice were treated for three days with streptomycin followed by a simultaneous challenge of *E. coli* O157:H7 orally, and mitomycin intraperitoneally (J. Fuji *et al.*, "Direct evidence of neuron impairment by oral infection with Verotoxin-producing *Escherichia coli* O157:H7 in mitomycin-treated mice," Infect. Immun., 62: 3447-34453 [1994]). All of the animals died within four days. Immunoelectron-microscopy strongly suggested that death was due to the toxic effects of VT2v (a structural variant of VT2), on both the endothelial cells and neurons in the central nervous system which resulted in fatal acute encephalopathy.

Wadolkowski *et al.* studied colonization of *E. coli* O157:H7 in mice. Mice were treated with streptomycin and fed 10^{10} *E. coli* O157:H7 (E. A. Wadolkowski *et al.*, "Mouse

model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun., 58: 2438-2445 [1990]; and E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun., 58: 3959-3965 [1990]). All of the mice died due to severe, disseminated, acute necrosis of proximal convoluted tubules. In mouse models, glomerular damage was not observed, but toxic acute renal tubular necrosis was observed which is characteristic of some HUS patients. The failure of mice to show glomerular damage is thought to be due to the absence of a functional globotriaosyl ceramide receptor specific for verotoxins in the glomeruli of the kidneys. Administration of VT2 subunit-specific monoclonal antibodies prior to infection prevented all pathology and death.

E. Current Therapeutic Approaches

E. coli O157:H7 disease is not adequately controlled by current therapy. Patient treatment is tailored to manage fluid and electrolyte disturbances, anemia, renal failure and hypertension. Although *E. coli* O157:H7 is susceptible to common antibiotics, the role of antibiotics in the treatment of infection has questionable merit. In both retrospective and prospective studies, prophylaxis or treatment with antibiotics such as trimethoprim-sulfamethoxazole, there was either no benefit or an increased risk of developing HUS (T. N. Bokete *et al.*, "Shiga-like toxin producing *Escherichia coli* in Seattle children: a prospective study." Gastroenterol., 105: 1724-1731 [1993]; A. T. Pavia *et al.*, "Hemolytic uremic-syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations." J. Pedatr., 116: 544-551 [1990]; F. Proulx *et al.*, "Randomized, controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis." J. Pediatr. 121: 299-303 [1992]; and A. L. Carter *et al.*, "A severe outbreak of *Escherichia coli* O157:H7-associated hemorrhagic colitis in a nursing home." New Eng. J. Med., 317: 1496-1500 [1987]).

The mechanisms by which antibiotics increase the risk of infection or related complications might involve enhancement of toxin production, release of toxins from killed organisms, or alteration of normal competing intestinal flora allowing for pathogen overgrowth (M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*." Clin. Microbiol. Rev., 2: 15-38 [1989]). A further concern in the use of antibiotics is the potential acquisition of antimicrobial resistance by *E. coli* O157:H7 (C. R. Dorn, "Review of foodborne outbreak of *Escherichia coli* O157:H7 infection in the western United States." JAVMA 203: 1583-1587 [1993]).

In addition, by the time symptoms are serious enough to attract medical attention, it is likely that verotoxins are already entering the systemic circulation or will do so shortly thereafter. Although antimicrobials may help to prevent pathology resulting from the action of toxin on the bowel lumen. However, by the time symptoms of HUS have developed, the patient has ceased shedding organisms. Thus, antimicrobial treatment during HUS disease is of less value, and often contraindicated, due to the increased risk of complications associated with administration of antimicrobials to patients susceptible to development of HUS. Importantly, there is currently no antitoxin commercially available for use in treating affected patients. What is needed is a means to block the progression of disease, without the complications associated with antimicrobial treatment.

DESCRIPTION OF THE DRAWINGS

Figure 1 is an SDS-PAGE of rVT1 and rVT2.

Figure 2 shows HPLC results for rVT1 and rVT2.

Figure 3 shows rVT1 and rVT2 toxicity in Vero cell culture.

Figure 4 shows EIA reactivity of rVT1 and rVT2 antibodies to rVT1.

Figure 5 shows EIA reactivity of rVT1 and rVT2 Antibodies to rVT2.

Figure 6 shows Western Blot reactivity of rVT1 and rVT2 antibodies to rVT's:

Panel 6A contains preimmune IgY;
Panel 6B contains rVT1 IgY; and
Panel 6C contains rVT2 IgY.

Figure 7 shows neutralization of rVT1 cytotoxicity in Vero cells.

Figure 8 shows neutralization of rVT2 cytotoxicity in Vero cells.

Figure 9 shows renal sections from *E. coli* O157:H7-infected mice treated with IgY

Panel 9A shows a representative kidney section from a mouse treated with preimmune IgY;
Panel 9B shows a representative kidney sections from a mouse treated with rVT1; and
Panel 9C shows a representative kidney section from a mouse treated with rVT2 IgY.

Figure 10 shows the fusion constructs of VT components and affinity tags.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of toxin polypeptides in a host cell, and indicates that the host cell is producing more of the toxin by virtue of the introduction of nucleic acid sequences encoding the toxin polypeptide than would be expressed by the host cell absent the introduction of these nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce the toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*i.e.*, an *E. coli* verotoxin and/or fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the *E. coli* protein as expressed in a host cell, may provide an "affinity tag" to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (*i.e.*, toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein, the term "affinity tag" refers to such structures as a "poly-histidine tract" or "poly-histidine tag," or any other structure or compound which facilitates the purification of a recombinant fusion protein from a host cell, host cell culture supernatant, or both. As used herein, the term "flag tag" refers to short polypeptide marker sequence useful for recombinant protein identification and purification.

As used herein, the terms "poly-histidine tract" and "poly-histidine tag," when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

As used herein, the term "chimeric protein" refers to two or more coding sequences obtained from different genes, that have been cloned together and that, after translation, act as

a single polypeptide sequence. Chimeric proteins are also referred to as "hybrid proteins." As used herein, the term "chimeric protein" refers to coding sequences that are obtained from different species of organisms, as well as coding sequences that are obtained from the same species of organisms.

As used herein, the term "protein of interest" refers to the protein whose expression is desired within the fusion protein. In a fusion protein, the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

As used herein, the term "maltose binding protein" and "MBP" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of substantially all immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins; the percent of recombinant toxin polypeptides is thereby increased in the sample.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell. is a protein which exists in solution in the cytoplasm of the host cell: if the protein contains a signal sequence. the soluble protein is exported to the periplasmic space in bacterial hosts and is secreted into the culture medium of eukaryotic cells capable of secretion or by bacterial hosts possessing the appropriate genes. In contrast. an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion bodies) in the host cell. High level expression (*i.e.*. greater than 1 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

A distinction is drawn between a soluble protein (*i.e.*. a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (*i.e.*. rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride. urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However. dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out): therefore. SDS-solubilized inclusion body protein is soluble but not refolded.

As used herein. the term "reporter reagent" or "reporter molecule" is used in reference to compounds which are capable of detecting the presence of antibody bound to antigen. For example. a reporter reagent may be a colorimetric substance which is attached to an enzymatic substrate. Upon binding of antibody and antigen. the enzyme acts on its substrate and causes the production of a color. Other reporter reagents include. but are not limited to fluorogenic and radioactive compounds or molecules.

As used herein the term "signal" is used in reference to the production of a sign that a reaction has occurred. for example. binding of antibody to antigen. It is contemplated that signals in the form of radioactivity. fluorogenic reactions. and enzymatic reactions will be

used with the present invention. The signal may be assessed quantitatively as well as qualitatively.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of *E. coli* toxin in a subject.

As used herein, the term "acute intoxication" is used in reference to cases of *E. coli* infection in which the patient is currently suffering from the effects of toxin (*e.g.*, *E. coli* verotoxins or enterotoxins). Signs and symptoms of intoxication with the toxin may be immediately apparent. Or, the determination of intoxication may require additional testing, such as detection of toxin present in the patient's fecal material.

As used herein, the term "at risk" is used in references to individuals who have been exposed to *E. coli* and may suffer the symptoms associated with infection or disease with these organisms, especially due to the effects of verotoxins.

SUMMARY OF THE INVENTION

The present invention relates to antitoxin therapy for humans and other animals. Antitoxins which neutralize the pathologic effects of *E. coli* toxins are generated by immunization of avian hosts with recombinant toxin fragments. In one embodiment, the present invention contemplates a method of treatment administering at least one antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable to an intoxicated subject. It is contemplated that the intoxicated subject will be either an adult or a child.

In a preferred embodiment, the *E. coli* verotoxin is recombinant. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence. In one embodiment of the *E. coli* fusion protein, the fusion protein comprises a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

Various routes of administration, are contemplated for providing the *E. coli* antitoxin(s) to an affected individual, including but not limited to, parenteral as well as oral routes of administration. In a particularly preferred embodiment, the route of administration is parenteral.

The present invention also includes the embodiment of a method of prophylactic treatment in which an antitoxin directed against at least one *E. coli* verotoxin in an aqueous

solution in therapeutic amount that is parenterally administrable. and is administered to at least one subject at risk of diarrheal disease. It one embodiment. the antitoxin is parenterally administered.

In one embodiment. the subject is at risk of developing extra-intestinal complications of *E. coli* infections. including but not limited to. hemolytic uremic syndrome. thrombotic thrombocytopenic purpura. etc.

The present invention also includes the embodiment of a composition which comprises neutralizing antitoxin directed against at least one *E. coli* verotoxin in an aqueous solution in therapeutic amounts. In one particularly preferred embodiment. the *E. coli* verotoxin is a recombinant toxin. In an alternative embodiment. the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT1 sequence. In another embodiment. the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT2 sequence. In yet another embodiment. the composition of the antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment. the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1. In an alternative embodiment. the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2. Indeed. the invention contemplates an antitoxin that is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment. the antitoxin is an avian antitoxin.

The present invention also comprises a method of treatment of enteric bacterial infections comprising administering an avian antitoxin directed against at least one verotoxin produced by *E. coli* in an aqueous solution in therapeutic amount. to at least one infected subject. In one preferred embodiment. the avian antitoxin is administered parenterally.

In another embodiment. the *E. coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7. O1:NM: O2:H5: O2:H7: O4:NM: O4:H10: O5:NM: O5:H16: O6:H1: O18:NM: O18:H7: O25:NM: O26:NM: O26:H11: O26:H32: O38:H21: O39:H4: O45:H2: O50:H7: O55:H7: O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM: O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10: O117:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM: O125:H8: O126:NM: O126:H8: O128:NM: O128:H2: O128:H8: O128:H12: O128:H25: O145:NM: O125:H25: O146:H21: O153:H25: O157:NM: O163:H19: O165:NM: O165:19: and O165:H25. In one embodiment. the antitoxin comprises antitoxin directed against at least one

Escherichia coli verotoxin. In another embodiment. the antitoxin is cross-reacts with at least one *Escherichia coli* verotoxin. In yet another embodiment. the antitoxin is reactive against toxins produced by members of the genus *Shigella*. including *S. dysenteriae*.

The present invention also contemplates uses for the toxin fragments in vaccines and diagnostic assays. The fragments may be used separately as purified. soluble antigens or. alternatively. in mixtures or "cocktails." The present invention thus comprises a method for detecting *Escherichia coli* verotoxin in a sample in which a sample.an antitoxin raised against *Escherichia coli* verotoxin. and a reporter reagent capable of binding the antitoxin are provided. The antitoxin is added to the sample. so that the antitoxin binds to the *E. coli* verotoxin in the sample. In one embodiment. the antitoxin is an avian antitoxin. In an alternative embodiment. the method further comprises the steps of washing unbound antitoxin from the sample. adding at least one reporter reagent to the sample. so that said reporter reagent binds to any antitoxin that is bound. washing the unbound reporter reagent from the sample and detecting the reporter reagent bound to the antitoxin bound to the *Escherichia coli* verotoxin. so that the verotoxin is detected. In one embodiment. the detecting is accomplished through any means. such as enzyme immunoassay. radioimmunoassay. fluorescence immunoassay. flocculation. particle agglutination. and *in situ* chromogenic assay. In one preferred embodiment. the sample is a biological sample. In an alternative preferred embodiment. the sample is an environmental sample.

DESCRIPTION OF THE INVENTION

The present invention contemplates treating humans and other animals intoxicated with at least one bacterial toxin. It is contemplated that administration of antitoxin will be used to treat patients effected by or at risk of symptoms due to the action of bacterial toxins. It is also contemplated that the antitoxin will be used in a diagnostic assay to detect the presence of toxins in samples. The organisms. toxins and individual steps of the present invention are described separately below.

I. Antibodies Directed Against *E. coli* and Associated Toxins

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against various *E. coli* serotypes. their toxins. enzymes or other metabolic by-products. cell wall components. or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization

of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens. Examples of these toxins include the verotoxins VT1 and VT2.

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin may be used as an effective therapeutic against one or more toxin(s) produced by other *E. coli* serotypes, or other toxin producing organisms (*e.g.*, *Shigella*, *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus mutans*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, other *Pseudomonas* species, *Vibrio* species, *Clostridium* species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

II. Obtaining Antibodies In Non-Mammals

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies may be obtained from non-mammals without immunization. In the case where no immunization is contemplated, the present invention may use non-mammals with preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

In a preferred embodiment, the method of the present invention contemplates immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (*i.e.*, recombinant toxin proteins). In a preferred embodiment, the immunogen comprises recombinant VT1 and/or VT2.

When immunization is used, the preferred non-mammal is from the class *Aves*. All birds are contemplated (*e.g.*, duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement (*See* H.N. Benson *et al.*,

J. Immunol. 87:616 [1961]). Thus, chicken antibody will normally not cause a complement-dependent reaction (A.A. Benedict and K. Yamaga, *"Immunoglobulins and Antibody Production in Avian Species," in Comparative Immunology* (J.J. Marchaloni, ed.), pp. 335-375, Blackwell, Oxford [1966]). Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins presently known.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum (*See* R. Patterson *et al.*, J. Immunol. 89:272 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 [1983]). In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is more pure and more homogeneous; there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. In one embodiment, glutaraldehyde treatment of the toxin is contemplated. In an alternative embodiment, formaldehyde treatment of the toxin is contemplated.

It is not intended that the present invention be limited to a particular mode of immunization; the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. As used herein, the term "adjuvant" is defined as a substance known to increase the immune response to other antigens when administered with other antigens. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of

adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. The invention also contemplates the use of fowl adjuvant commercially available from RIBI. as well as Quil A adjuvant commercially available from Accurate Chemical and Scientific Corporation. and Gerbu adjuvant also commercially available (GmDP: C.C. Biotech Corp.).

When immunization is used. the present invention contemplates a wide variety of immunization schedules. In one embodiment. a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly. it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 35.

Where birds are used and collection of antibody is performed by collecting eggs. the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be buffer-extracted and used analytically. While unpurified. this preparation can serve as a reference for activity of the antibody prior to further manipulations (*e.g.*. immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

When purification is used. the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically. the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple. inexpensive procedures. In one embodiment. chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000 (Polson *et al.*. Immunol. Comm. 9:495 [1980]). The technique is rapid. simple. and relatively inexpensive and yields an immunoglobulin fraction that is significantly more pure. in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed.

PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

IV. Treatment

The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by parenteral administration of antitoxin.

A. Dosage Of Antitoxin

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin. but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (*e.g*, horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies; and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when. as noted above. the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment. the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived. PEG-purified antibody as antitoxin allows for the administration of: 1) non (mammalian)-complement-fixing. avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

As is true in cases of botulism. the degree of an individual's exposure to *E. coli* toxin and the prognosis are often difficult to assess. and depend upon a number of factors (*e.g.*, the quantity of contaminated food ingested. the toxigenicity and serotype of *E. coli* strain ingested. etc.). Thus. the clinical presentation of a patient is usually a more important consideration than a quantitative diagnostic test. for determination of dosage in antitoxin

administration. Indeed, for many toxin-associated diseases (*e.g.*, botulism, tetanus, diphtheria, etc.), there is no rapid, quantitative test to detect the presence of the toxin or organism. Rather, these toxin-associated diseases are medical emergencies which mandate immediate treatment. Confirmation of the etiologic agent must not delay the institution of therapy, as the condition of an affected patient may rapidly deteriorate. In addition to the initial treatment with antitoxin, subsequent doses may be indicated, as the patient's disease progresses. The dosage and timing of these subsequent doses is dependent upon the signs and symptoms of disease in each individual patient.

It is contemplated that the administration of antitoxin to an affected individual would involve an initial injection of an approximately 10 ml dose of immune globulin (with less than approximately 1 gram of total protein). In one preferred embodiment, it is contemplated that at least 50% of the initial injection comprises immune globulin. It is also contemplated that more purified immune globulin be used for treatment, wherein approximately 90% of the initial injection comprises immune globulin. When more purified immune globulin is used, it is contemplated that the total protein will be less than approximately 100 milligrams. It is also contemplated that additional doses be given, depending upon the signs and symptoms associated with *E. coli* verotoxin disease progression.

B. Delivery Of Antitoxin

Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is parenteral or oral.

In one embodiment, antitoxin is parenterally administered to a subject in an aqueous solution. It is not intended that the parenteral administration be limited to a particular route. Indeed, it is contemplated that all routes of parenteral administration will be used. In one embodiment, parenteral administration is accomplished via intramuscular injection. In an alternative embodiment, parenteral administration is accomplished via intravenous injection.

In another embodiment, antitoxin is delivered in a solid form (*e.g.*, tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (*e.g.*, carbonate buffer, pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an

aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant or a dietary supplement formula (*e.g.*, Similac®, Ensure®, and Enfamil®). Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

Methods of applying enteric coatings to pharmaceutical compounds are well known to the art (companies specializing in the coating of pharmaceutical compounds are available: for example, The Coating Place [Verona, WI] and AAI [Wilmington, NC]). Enteric coatings which are resistant to gastric fluid and whose release (*i.e.*, dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available (for example, the polymethacrylates Eudragit® L and Eudragit® S [Röhm Tech Inc., Malden, MA]). Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause them to dissolve thereby releasing the antitoxin.

The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin. In another embodiment of treatment of acute intoxication, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (*i.e.*, a suspension) to recipients unable to tolerate administration of tablets (*e.g.*, children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic

dosage. In yet another preferred embodiment of prophylactic treatment. a therapeutic dosage of the antitoxin in a delivery solution. is parenterally administered.

V. Multivalent Vaccines Against *E. coli* Strains

The invention contemplates the generation of multivalent vaccines for the protection of an organism (particularly humans) against several *E. coli* strains. Of particular interest is a vaccine which stimulates the production of a humoral immune response to *E. coli* O157:H7. O26:H11. O113:H21. O91:H21. and O111:NM. in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the *E. coli* serotypes listed above. When native toxin proteins are used as immunogens they are generally modified to reduce the toxicity. It is contemplated that glutaraldehyde-modified toxin proteins will be used. In an alternative embodiment. is formaldehyde-modified toxin proteins will be used.

The invention contemplates that recombinant *E. coli* verotoxin proteins be used in conjunction with either native toxins or toxoids from other organisms as antigens in a multivalent vaccine preparation. It is also contemplated that recombinant *E. coli* toxin proteins be used in the multivalent vaccine preparation.

VI. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture (*e.g.*. microbiological cultures). On the other hand. it is meant to include both biological and environmental samples.

Biological samples may be animal. including human. fluid. solid (*e.g.*. stool) or tissue. as well as liquid and solid food and feed products and ingredients such as dairy items. vegetables. meat and meat by-products. and waste. Biological samples may be obtained from all of the various families of common domestic animals. including but not limited. to bovines (*e.g.* cattle). ovines (*e.g.*. sheep). caprines (*e.g.*. goats). porcines (*e.g.*. swine). equines (*e.g.*. horses). canines (*e.g.*. dogs). lagamorphs (*e.g.*. rabbits). and felines (*e.g.*. cats). etc. It is also intended that samples may be obtained from feral or wild animals. including. but not limited to. such animals as ungulates (*e.g.*. deer). bear. fish. lagamorphs. rodents. etc.

Environmental samples include environmental material such as surface matter. soil. water and industrial samples. as well as samples obtained from food and dairy processing

instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin VT1 and toxin VT2 proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (*e.g.*, a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (*e.g.*, microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (*e.g.*, soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following

the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (*i.e.*, in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (*e.g.*, the addition of enzyme substrate in enzyme systems; observation using fluorescent light for fluorescent dye systems; and quantitation of radioactivity for radioactive systems).

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); rpm (revolutions per minute); BSA (bovine serum albumin); ELISA (enzyme-linked immunosorbent assay); IgG (immunoglobulin G); IgY (immunoglobulin Y); IP (intraperitoneal); SC (subcutaneous); H_2O (water); HCl (hydrochloric acid); LD_{100} (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography); Kda (kilodaltons); gm (grams); μg (micrograms); mg (milligrams); ng (nanograms); μl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); μm (micrometer); M (molar); mM (millimolar); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); $MgCl_2$ (magnesium chloride); NaCl (sodium chloride); Na_2CO_3 (sodium carbonate); OD_{280} (optical density at 280 nm); OD_{600} (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); Amicon (Amicon, Inc., Beverly, MA); Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologics Laboratory, (a division of Becton Dickinson), Cockeysville, MD); Becton Dickinson (Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA); Biotech (C-C Biotech Corp., Poway, CA); Charles River (Charles River Laboratories, Wilmington, MA); Falcon (*e.g.* Baxter Healthcare Corp., McGaw Park, IL and Becton Dickinson); Fisher Biotech (Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL, Grand Island, NY); Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL);

Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs, Inc., Beverly, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia (Pharmacia, Inc., Piscataway, NJ); Qiagen (Qiagen, Chatsworth, CA); Showdex (Showa Denko America, Inc., New York, NY); Sigma (Sigma Chemical Co., St. Louis, MO); RIBI (RIBI Immunochemical Research Inc., Hamilton, MT); Accurate Chemical and Scientific Corp. (Accurate Chemical and Scientific Corp., Hicksville, NY); Kodak (Eastman-Kodak, Rochester, NY); and Stratagene (Stratagene, La Jolla, CA).

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. The specification gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

The first set of Examples (Examples 1-5) was designed to develop an antidote to *E. coli* O157:H7 verotoxins and evaluate its effectiveness *in vitro* and *in vivo*. In the first experiments, high titer verotoxin antibodies were generated in laying hens hyperimmunized with chemically detoxified and/or native verotoxins. These Laying hens were immunized with either recombinant *E. coli* O157:H7 VT1 or VT2 (rVT1 and rVT2) treated with glutaraldehyde and mixed with adjuvant.

Next, toxin-reactive polyclonal antibodies were isolated by bulk fractionation from egg yolks pooled from hyperimmunized hens. Large quantities of polyclonal antibodies (IgY) were harvested from resulting eggs using a two-step polyethylene glycol fractionation procedure.

Third, the immunoreactivity and yields of VT IgY were analyzed by analytical immunochemical methods (*e.g.*, enzyme immunoassay (EIA) and Western blotting). EIA and Western blot analysis showed that the resulting egg preparations contained high titer IgY that reacted with both the immunizing and the heterologous toxins (*i.e.*, rVT1 IgY reacted against both rVT1 and rVT2, and vice versa).

Fourth, VT neutralization potency was analyzed *in vitro* using a Vero cytotoxicity assay. Vero cytotoxicity of rVT1 and rVT2 could be completely inhibited by VT IgY. These antibodies also demonstrated substantial verotoxin cross-neutralization.

Fifth, the efficacy of passively administered avian verotoxin antibodies in preventing the lethal effects of verotoxin poisoning was assessed in a mouse disease model. Toxin neutralizing antibodies were administered by parenteral dosing regimens to assess the most

effective strategy for therapeutic intervention. Efficacy of verotoxin antibodies was demonstrated using multiple murine disease models. In these experiments, antibodies prevented both the morbidity and lethality of homologous and heterologous toxins using a toxin/antitoxin premix format: mice infected orally with a lethal dose of viable *E. coli* O157:H7 were protected from both morbidity and lethality when treated parenterally four hours post-infection with either rVT1 or rVT2 antibodies: and mice given a lethal dose of *E. coli* O91:H21 (a particularly virulent strain which only produces VT2c, a VT2 structural variant) and treated parenterally *up to 10 hours later* with rVT1 IgY administered parenterally were protected from both morbidity and lethality.

EXAMPLE 1

TOXIN ANALYSIS AND IMMUNIZATION

Purified recombinant *E. coli* O157:H7 verotoxins, rVT1 and rVT2, were obtained from Denka Sieken Co., Ltd. (Tokyo, Japan). Toxin genes were isolated, inserted into expression plasmids, and expressed in *E. coli*. Recombinant proteins were then purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE Sephacryl and hydroxyapatite, and gel filtration chromatography by the supplier. Upon receipt, toxins were analyzed to verify identity, purity and toxicity, as described below.

A. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Samples of each toxin (2 μg) were heat-denatured in a buffer containing SDS and β-mercaptoethanol followed by electrophoresis on 10–20% gradient gels (Bio-Rad, Richmond, CA). Resolved polypeptide bands were visualized using the silver stain procedure of C.R. Merril, *et al.*, "Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins." Science 211: 1437-1438 (1981).

VT1 and VT2 are each composed of subunit A and multiple copies of subunit B. Subunit A is often nicked into fragments A1 and A2 which are linked by a disulfide bridge. As shown in Figure 1, when separated by SDS-PAGE in the presence of β-mercaptoethanol, rVT1 resolved into 3 bands that corresponded to subunit A (~31 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~4 Kda). Similarly, rVT2 resolved into subunit A (~33 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~8 Kda) (Figure 1). In this Figure, rVT1 is in Lane 1, and rVT2 is in Lane 2; the positions of

molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

These results are consistent with previous reports of VT1 and VT2 purified from naturally occurring toxigenic strains (V. V. Padhye *et al.*. "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." Biochem. Biophys. Res. Commun.. 139: 424-430 [1986]; and F. B. Kittel *et al.*. "Characterization and inactivation of verotoxin 1 produced by *Escherichia coli* O157:H7." J. Agr. Food Chem., 39: 141-145 [1991]).

B. High Performance Liquid Chromatography (HPLC).

Chromatography was performed at room temperature (RT) under isocratic conditions using a Waters 510 HPLC pump. Eluted protein was measured using a Waters 490E programmable multi-wavelength detector (Millipore Corp.. Milford. MA). The VT's were separated on an 8 x 300 mm (ID) Shodex KW803 column. using 10 mM sodium phosphate. 0.15 M NaCl. pH 7.4 (phosphate buffered saline [PBS]) as the mobile phase at a flow rate of 1 ml/min.

The purity of non-denatured rVT's was assessed by HPLC. As shown in the chromatographs in Figure 2. each toxin eluted at approximately 10 min. as a single absorbance peak at 280 nm. By integration of the area under each peak. the rVT's were shown to be >99% pure.

C. Vero Cell Cytotoxicity Assay.

Cytotoxic activity of rVT1 and rVT2 was assessed using modified procedures of Padhye. *et al.* (V. V. Padhye *et al.*. "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." Biochem. Biophys. Res. Commun.. 139: 424-430 [1986]). and McGee. *et al.*, (Z. A. McGee. *et al.*. "Local induction of tumor necrosis factor as molecular mechanism of mucosal damage by gonococci." Microbial Pathogenesis 12: 333-341 [1992]). Microtiter plates (96 well. Falcon. Microtest III) were inoculated with approximately 1 x 10^4 Vero cells (ATCC. CCL81) per well (100 μl) and incubated overnight at 37°C in the presence of 5% CO_2 to form Vero cell monolayers. rVT1 and rVT2 solutions were serially diluted in Medium 199 supplemented with 5% fetal bovine serum (Life Technologies. Grand Island. NY). added to each well of the microtiter plates and incubated at 37°C for 18–24 hrs. Adherent (viable) cells were stained with 0.2% crystal

violet (Mallinckrodt) in 2% ethanol. Excess stain was rinsed away and the stained cells were solubilized by adding 100 µl of 1% SDS to each well. Absorbance of each well was measured at 570 nm, and the percent cytotoxicity of each test sample was calculated using the following formula:

$$\% \text{ Vero Cytotoxicity} = [1 - (\text{Absorbance Sample}/\text{Absorbance Control})] \times 100$$

To determine whether the rVT's possessed potency equivalent to published cytotoxicity values, a Vero cell cytotoxicity assay was performed (Figure 3). Between 0.01–10,000 pg of either rVT1 or rVT2 was added to Vero cells. The amounts of rVT causing 50% cell death (CD_{50}), as calculated by second degree polynomial curve fitting were 0.97 pg and 1.5 pg, for rVT1 and rVT2, respectively. These results are consistent with CD_{50} values reported previously for naturally occurring VT1 and VT2 in the range 1–35 pg and 1–25 pg, respectively (M. Petric *et al.*, Purification and biological properties of *Escherichia coli* verocytotoxin." FEMS Microbiol. Lett., 41: 63-68 [1987]; V. L. Tesh, *et al.*, "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun., 61: 3392-3402 [1993]; N. Dickie *et al.*, "Purification of an *Escherichia coli* Serogroup O157:H7 verotoxin and its detection in North American hemorrhagic colitis isolates." J. Clin. Microbiol., 27: 1973-1978 [1989]; and U. Kongmuang, *et al.*, "A simple method for purification of Shiga or Shiga-Like toxin from *Shigella dysenteriae* and *Escherichia coli* O157:H7 by immunoaffinity chromatography." FEMS Microbiol. Lett., 48: 379-383 [1987]). It has been observed that toxicity is lost with storage, explaining why higher amounts of toxin were used in the neutralization assays described below.

D. Mouse Lethal Dose Determination.

To verify rVT1 and rVT2 toxicity, male (20–22 g) CD-1 mice were injected intraperitoneally with varying amounts of rVT1 or rVT2 in 200 µL phosphate buffer. Doses were selected based on published LD_{50} values for VT1 and VT2 in CD-1 mice. To minimize the sacrifice of live animals, a full statistical toxin LD_{50} was not determined. Mice were observed for morbidity and mortality over 7-day period.

Further confirmation of rVT toxicity was obtained from mouse lethality experiments (Table 2). Mice were injected intraperitoneally with varying amounts of either rVT1 or rVT2 and observed 7 days for mortality. Within 72–120 hrs. post-injection, all of the mice died

from 100 ng of rVT1 or 10 ng of rVT2, respectively. This lethality study served as a verification of expected toxicity but not as a statistical determination of LD_{50}. Nonetheless, these results were consistent with toxicity studies which reported LD_{50} values in CD-1 mice of 0.4–2.0 μg for purified VT1 and 0.001–1.0 μg for purified VT2 (V. L. Tesh, *et al.*, "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun., 61: 3392-3402 [1993]; and A. D. O'Brien, and G. D. LaVeck, "Purification and characterization of *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*." Infect. Immun. 40: 675-683 [1983]).

Table 2.
Lethality of rVT1 in CD-1 Mice

ng VT1 Injected	Survivors/Total	Hours Post-Injection
100	7/7	24 ± 2
	5/7	48 ± 2
	0/7	72 ± 2
10	7/7	24 ± 2
	7/7	48 ± 2
	7/7	72 ± 2
1.0	6/6	24 ± 2
	6/6	48 ± 2
	6/6	72 ± 2

Table 3.
Lethality of rVT2 in CD-1 Mice

ng VT2 Injected	Survivors/Total	Hours Post-Injection
10	3/6	48 ± 2
	2/6	72 ± 2
	0/6	120 ± 2
1.0	5/6	48 ± 2
	4/6	72 ± 2
	0/6	120 ± 2
0.1	6/6	48 ± 2
	6/6	72 ± 2
	6/6	120 ± 2

The recombinant toxins used in these studies thus appeared to contain protein components and toxicities consistent with literature reports for native toxins. Based on these structural and functional analyses, the rVT's were considered suitable as antigens to generate specific avian antibodies.

E. Antigen Preparation.

Lyophilized samples, rVT1 and rVT2 were received and each was reconstituted with 2.5 mL of deionized water to a final concentration of 100 μg/ml in phosphate buffer. To form a toxoid, the solutions were then treated with 0.4% glutaraldehyde (Mallinckrodt) at 4°C overnight and stored at -20°C thereafter. When needed, toxoid was thawed and mixed 5:1 (volume:volume) with GERBU adjuvant (C. C. Biotech Corporation, Poway, CA). White Leghorn laying hens were injected subcutaneously with 25 μg of either rVT1 or rVT2 toxoid in adjuvant at 2–3 week intervals.

EXAMPLE 2
PEG EXTRACTION OF EGG YOLK ANTIBODY

Hyperimmune eggs were collected after 3 immunizations with toxoid. Egg yolks were separated from whites, pooled according to their immunogen group and blended with 4 volumes of 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). Polyethylene glycol

8000 (PEG) (Amresco. Solon. OH) was then added to a final concentration of 3.5% and the mixture centrifuged at 10.000 x g for 10 min. to remove the precipitated lipid fraction. IgY-rich supernatant was filtered through cheesecloth and PEG was again added to a final concentration of 12%. The solution was centrifuged as above and the resulting supernatant discarded. The IgY pellet was then dissolved in PBS to either the original (1X PEG IgY) or ¼ of the original (4X PEG IgY) yolk volume. filtered through a 0.45 μ membrane and stored at 4°C.

EXAMPLE 3
ANTITOXIN IMMUNOASSAYS

A. Enzyme Immunoassay (EIA).

EIA was used to monitor antibody responses during the immunization course. Wells of 96-well Pro-Bind microtiter plates (Falcon. through Scientific Products. McGaw Park. IL) were each coated with 1 μg of rVT's (not toxoid) in PBS overnight at 2–8°C. Wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound antigen. and the remaining protein binding sites were blocked with PBS containing 1 mg/ml BSA for 60 min. at room temperature (RT). IgY. diluted in PBS. was then added to the wells and incubated for 1 hr. at 37°C. Wells were washed as before to remove unbound primary antibody and incubated for 1 hr. at 37°C with alkaline phosphatase-conjugated rabbit-anti-chicken IgG (Sigma Chemical Company. St. Louis. MO) diluted 1:1000 in PBS-T. Wells were again washed and 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Company. St. Louis. MO) in 50 mM Na_2CO_3 10 mM $MgCl_2$, pH 9.5 was added and allowed to incubate at RT. Phosphatase activity was detected by absorbance at 410 nm using a Dynatech MR700 microtiter plate reader.

Laying Leghorn hens were immunized as described above (Example 1. part E). using glutaraldehyde-treated rVT's. Following several immunizations. eggs were collected and IgY harvested by PEG fractionation. Figures 4 and 5 show rVT1 or rVT2 specific antibody responses detected using EIA at dilutions of the original yolk IgY concentration of 1:30.000 and 1:6.000. respectively. IgY fractionated similarly from unimmunized hens (*i.e.*. preimmune antibody) did not react with either antigen at test dilutions above 1:50. Although these EIA results indicate significant antibody responses. prior experience with other toxin antigens has shown that optimization of immunization regimens. including increasing the amount of

antigen. can yield titers in excess of 1:100.000 (B. S. Thalley. *et al.*."Development of an Avian Antitoxin to Type A Botulinum Neurotoxin." *in* Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects. B. R. DasGupta. (ed.) [Plenum Press. New York. 1993] pp. 467-472). As may be expected due to their structural homology and consistent with previous reports (*e.g.*, V. V. Padhye *et al.*. "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7." *J. Agr. Food Chem.*. 39: 141-145 [1989]; S. C. Head *et al.*. "Purification and characterization of verocytotoxin 2." FEMS Microbiol. Lett.. 51: 211-216 [1988]; and N. C. Strockbine *et al.*. "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*." Infect. Immun.. 50: 695-700 [1985]). Figures 4 and 5 also demonstrate that antibodies generated against one toxin cross-reacted *in vitro* with the other toxin.

B. Western Blot Analysis.

Western blots (Figure 6) performed to determine the reactivity of rVT antibodies against constituent VT polypeptides showed that rVT1 and rVT2 antibodies reacted with subunit A and fragment A1 of either toxin. and with subunit B and fragment A2 of rVT1 only. In this Figure. Panel A contains preimmune IgY. Panel B contains rVT1 IgY. and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 (2μg) and Lane 2 contains rVT2 (2 μg). Preimmune IgY was largely nonreactive to either rVT. Both rVT IgY preparations. however. failed to react with subunit B and fragment A2 of rVT2. Some explanations for this lack of measurable reactivity might include poor immunogenicity. denaturation of the immunogen during glutaraldehyde treatment. loss of conformational epitopes due to detergent or reducing agent. or poor transfer to nitrocellulose.

To resolve the high and low molecular weight components. 2 μg each of rVT1 and rVT2 were separated by SDS-PAGE (described above) and then transferred to nitrocellulose paper using the Milliblot-SDE system (Millipore. Medford. MA) according to the manufacturer's instructions. Paper strips were stained temporarily with Ponceau S (Sigma Chemical Company. St. Louis. MO) to visualize the polypeptides and then blocked overnight in PBS containing 5% dry milk. Each strip was agitated gently in IgY diluted in PBS-T for 2 hrs. at RT. Strips were each washed with three changes of PBS-T to remove unbound primary antibody and incubated for 2 hrs. at RT with goat anti-chicken alkaline phosphatase (Kirkegaard and Perry. Gaithersburg. MD) diluted 1:500 in PBS-T containing 1 mg/ml BSA. The blots were washed as before and rinsed in 50 mM Na_2CO_3. pH 9.5. Strips were

submerged in alkaline-phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Kirkegaard and Perry) until sufficient signal was observed. Color development was stopped by flooding the blots with water.

EXAMPLE 4
IN VITRO TOXIN NEUTRALIZATION: VERO CELL ASSAY

IgY neutralization of rVT1 and rVT2 was assessed using the modified Vero cytotoxicity assay described above (Example 1, part C). Various concentrations of IgY, diluted in Medium 199 supplemented with 5% fetal bovine serum (GIBCO), were mixed with sufficient toxin to cause 50% cell death and allowed to incubate at 37°C for 60 minutes. These toxin/antibody mixtures were then added to Vero cell-coated microtiter plate wells according to the procedure described above (Example 1, part C).

The toxin neutralization capacity of the rVT antibodies was analyzed first using a Vero cell toxicity assay. The results in Figure 7 show that rVT1 IgY neutralized completely the cytotoxic activity of rVT1 at an endpoint dilution of 1/320. Furthermore, rVT2 IgY neutralized the heterologous rVT1 toxin, but at a higher endpoint concentration.

In a similar experiment (see Figure 8), rVT1 and rVT2 antibodies were each able to neutralize rVT2 at equivalent endpoint dilutions. This strong cross-neutralization correlates with the observed strong cross-reactivity of VT1 IgY with VT2 A seen on Western blots (Figure 6). These results show that IgY antibodies are able to neutralize effectively VT cytotoxicity and that the antibodies can cross-neutralize structurally-related heterologous toxins.

EXAMPLE 5
TOXIN NEUTRALIZATION: MOUSE ASSAYS

A. Toxin Challenge Model.

IgY in PBS was premixed with a lethal dose of toxin (as determined above) and injected intraperitoneally into male CD-1 (20–22 gm) mice. Mice were observed for a 7-day period for signs of intoxication such as ruffled fur, huddling and disinclination to move, followed by hind leg paralysis, rapid breathing and death. Untreated, infected mice usually died within 12 hrs. after signs of severe illness (*i.e.*, within 48–72 hrs. post-injection).

Once it was demonstrated that rVT antibodies were able to neutralize rVT cytotoxicity *in vitro*, protection experiments were next performed in mice. First, animals were challenged with rVT premixed with rVT IgY to determine whether toxin lethality could be neutralized under conditions optimal for antigen/antibody reaction. Tables 4 and 5 show that antibodies premixed with the homologous toxin (*e.g.*, rVT1 with rVT1 IgY) prevented lethality of rVT. Preimmune IgY was unable to neutralize either toxin in these studies.

Table 4

Neutralization of rVT1 Using rVT IgY

100 ng rVT2 Premixed*	Survivors/Total	*p*
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

*Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Table 5

Neutralization of rVT2 Using rVT IgY

10 ng rVT1 Premixed*	Survivors/Total	*p*
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

*Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Antibodies premixed with the heterologous toxin (*e.g.*, rVT2 with rVT1 IgY) also prevented lethality *in vivo*. These data are in contrast to previous observations where rabbit polyclonal antibodies generated against either toxin were cross-reactive with the heterologous toxin by EIA and Western blot, but were unable to neutralize the heterologous toxin in either Vero cell cytotoxicity and mouse lethality assays (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," FEMS Microbiol. Lett., 51: 211-216

[1988]; N. C. Strockbine *et al.*, "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*," Infect. Immun., 50: 695-700 [1985]; and V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7," Biochem. Biophys. Res. Commun., 139: 424-430 [1986]).

However, Head *et al.*, showed that VT2 B-subunit specific monoclonal antibodies neutralized VT1 weakly in a Vero cytotoxicity assay (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]). In a report by Donohue-Rolfe, *et al.*, a VT2 B subunit-specific monoclonal antibody neutralized both VT1 and VT2 completely in a Hela cytotoxicity assay (A. Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies," Infect. Immun., 57: 3888-3893 [1989]).

These results showed for the first time complete cross-neutralization in Vero cell cytotoxicity and mouse lethality assays, revealing that VT1 and VT2 do indeed share common neutralizing epitopes. These results may indicate that hens generate different antibody specificities as compared to mammals, and/or that differences in immunization methods might have maintained the immunogenicity of conformational epitopes necessary for cross-neutralization. Nonetheless, this cross-neutralization suggests that IgY antibodies may contain the range of reactivities essential for an effective antitoxin.

B. Viable organism infection model.

Streptomycin-resistant *E. coli* O157:H7 (strain 933 cu-rev) or *E. coli* O91:H21 (strain B2F1) (both kindly provided by Dr. Alison O'Brien, Dept. of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD) were used in a murine infection model described by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun., 58: 2438-2445 [1990]). Organisms were grown in Luria broth and incubated overnight at 37°C in an Environ Shaker (Lab Line, Melrose Park, IL) (T. Maniatis *et al.*, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., [1982]). Bacterial suspensions were centrifuged at 6700 x g for 5 minutes. The resulting pellet was then washed twice with sterile PBS and resuspended in sterile 20% (w/v) sucrose. Five to 8 week-old male CD-1 mice were provided drinking water containing 5 mg/ml streptomycin sulfate *ad libitum* for 24 hrs. Food and water were then withheld for

another 16–18 hrs. after which mice were challenged orally with 10^{10} streptomycin-resistant *E. coli* O157:H7 or O91:H21. Mice were housed individually and permitted food and water containing 5 mg/ml streptomycin sulfate. IgY was injected intraperitoneally at varying times post-infection and animals observed for both morbidity and mortality for 10 days.

To monitor bacterial colonization in animals. 1 gram of feces was collected. homogenized. and plated onto MacConkey agar medium (Difco Laboratories. Detroit. MI) containing 100 µg/ml streptomycin and incubated at 37°C as described by Wadolkowski. *et al.* (E. A. Wadolkowski *et al.*. "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun.. 58: 2438-2445 [1990]). The serotype of *E. coli* O157:H7. 933 cu-rev excreted in feces was confirmed by slide agglutination with O– and H–specific antisera (Difco Laboratories. Detroit. MI).

Kidneys were removed from experimental animals and fixed in 10% buffered neutral formalin. Sections of parafilm-embedded tissue were stained with hematoxylin and eosin (General Medical Laboratories. Madison. WI) and examined by light microscopy. All tissue sections were coded to avoid bias before microscopic examination to determine renal pathology.

The toxin neutralization ability of rVT IgY was further studied using a streptomycin-treated CD-1 mouse infection model. This model was chosen because it produces definitive systemic pathology and reproducible mortality.

In contrast to previous studies by Wadolkowski. *et al.* (E. A. Wadolkowski *et al.*. "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]). where mice were given subunit-specific monoclonal antibodies *prior* to infection. the mice in this study were inoculated orally with 2 x 10^{10} viable *E. coli* O157:H7 (strain 933 cu-rev) and treated with rVT IgY 4 hrs. *following* inoculation. Fecal cultures showed that 10^7–10^8 challenge organisms per gram of feces were shed throughout the course of the experiment. thus confirming that infection was established. Tables 6 and 7 show that animals treated with either rVT1 or rVT2 IgY were protected from lethality caused by infection ($p<0.01$ and $p<0.001$. respectively) and that preimmune IgY failed to provide protection to the mice.

Table 6
Protection of Mice From *E. coli* O157:H7
With rVT1 IgY

IgY Treatment	Survivors/Total	*p*	Morbidity/Total
Preimmune Antibody	0/5		5/5
rVT1 Antibody	9/10	< 0.01	1/10

*IgY was administered intraperitoneally 4 hours following infection. and once daily for 10 days thereafter.

Table 7
Protection of Mice From *E. coli* O157:H7
With rVT2 IgY

IgY Treatment	Survivors/Total	*p*	Morbidity/Total
Preimmune Antibody	0/6		6/6
rVT2 Antibody	10/10	< 0.005	0/10

*IgY was administered intraperitoneally 4 hours following infection. and once daily for 10 days thereafter.

Renal histopathology (*see* Figure 9) of the control (preimmune IgY) animals showed dilation. degeneration and renal tubular necrosis with no glomerular damage. This is consistent with previous reports showing that renal tubular involvement occurs predominantly in this streptomycin-treated mouse infectivity model (E. A. Wadolkowski *et al.*. "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]). Importantly. none of the survivors exhibited similar signs of morbidity though treated with IgY 4 hrs. *after* infection (*see* Figure 9).

Furthermore. avian antibodies generated against rVT1 were able to prevent both mortality and morbidity in a mouse model where VT2 alone is implicated in the pathogenesis and lethality of *E. coli* O157:H7 strain 933 cu-rev (E. A. Wadolkowski *et al.*. "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]).

To assess the broader utility of the IgY verotoxin antibodies in treating VTEC-associated disease. the mouse infectivity study was performed using a more virulent VTEC serotype known to produce VT2c—a structural variant of VT2—but not VT1 (S. W. Lindgren

et al., "Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model." Infect. Immun., 61: 3832-3842 [1993]).

Mice were inoculated orally with 5 x 10^9 *E. coli* O91:H21 (strain B2F1) and treated subsequently with IgY. Notably, the heterologous rVT1 IgY protected strongly against the lethal effects of the VT2c structural variant, even when administered as long as 10 hrs. following infection (Table 8). Ten hours was the longest treatment window tested in this study. Only 1 of the 8 animals treated with rVT1 IgY died (p <0.02), and those that survived showed no overt signs of renal histopathology (*i.e.*, acute bilateral tubular necrosis). It can thus be concluded that rVT1 IgY completely neutralized toxicity of VT2c, indicating its potential as a therapeutic for at least one other pathogenic VTEC.

Table 8
Protection of Mice From *E. coli* O91:H21
With rVT1 IgY

IgY Treatment	Survivors/Total	*p*	Morbidity/Total
Preimmune Antibody	0/7		7/7
rVT1 Antibody	7/8	< 0.02	1/8

*IgY was administered intraperitoneally 10 hours following infection, and once daily for 8 days thereafter.

These Examples highlight several important findings supporting the feasibility of using verotoxin antitoxin. First, polyclonal IgY generated against either VT1 or VT2 from *E. coli* O157:H7, cross-reacted with and fully cross-neutralized the toxicity of the non-immunizing toxin both *in vitro* and *in vivo*. Second, recombinant toxins fully neutralized the toxicity of naturally-occurring toxins produced by *E. coli* O157:H7 during the course of infection. Third, antibodies generated against rVT1 from *E. coli* O157:H7 could prevent morbidity and mortality in mice infected orally with lethal doses of *E. coli* O91:H21, a particularly virulent strain which only produces VT2c, suggesting their utility in preventing systemic sequelae. Because VT1 is identical to Shiga-toxin (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]), VT antibodies may also be useful in preventing complications stemming from *Shigella dysenteriae* infection. Finally, animals treated with VT

IgY were protected against both death and kidney damage when treated as long as 10 hrs. after infection, supporting the hypothesis that a window for antitoxin intervention exists.

These studies strongly support the use of parenterally-administered, toxin-specific IgY as a antitoxin to prevent life-threatening complications associated with *E. coli* O157:H7 and other VTEC infections. It is contemplated that this approach would be most useful in preventing HUS and other complications when administered after the onset of bloody diarrhea and before the presentation of systemic disease.

The VT IgY developed in these studies were shown to react with and neutralize both recombinant and naturally-occurring VT. The antibody titers as measured by EIA are indicative of reasonable antibody production in the hen, however much higher production levels can be obtained with larger immunizing doses.

The results from these Examples clearly demonstrate the feasibility and provide the experimental basis for development of an avian antidote for *E. coli* O157:H7 verotoxins suitable for use in humans. In contrast to previous reports showing that rabbit polyclonal VT1 and VT2 antibodies cross-reacted, but did not cross-neutralize the heterologous toxin in Vero cytotoxicity or in mouse lethality studies (*e.g.*, V. V. Padhye *et al.*, "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7," J. Agr. Food Chem., 39: 141-145 [1989]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," FEMS Microbiol. Lett., 51: 211-216 [1988]; and N. C. Strockbine *et al.*, "Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*," Infect. Immun., 50: 695-700 [1985]), these data provide the first demonstration of cross-neutralization *in vivo*. Antibodies against one toxin neutralized completely the heterologous toxin in both Vero cytotoxicity and mouse lethality assays. Both rVT1 and rVT2 antibodies also prevented morbidity (as assessed by renal histopathology) and mortality in mice infected with lethal doses of *E. coli* O157:H7 – the etiologic agent in 90% of the documented cases of hemolytic uremic syndrome (HUS) in the U.S. (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1990]). With at least two other VTEC serotypes known to cause HUS, the finding that rVT1 antibodies neutralized a VT2 variant produced by *E. coli* O91:H21 suggests that avian polyclonal antibodies may provide an effective antidote against other verotoxin-producing *E. coli*. These data also show for the first time, that antibodies may be administered *after* infection and still protect against morbidity and mortality.

EXAMPLE 6

EXPRESSION OF TOXIN GENES

The previous Examples clearly showed that avian polyclonal antibodies to recombinant toxins protected animals infected with verotoxigenic *E. coli*. This Example includes expression of toxin genes (A and B subunits alone and together as whole toxins) in suitable prokaryotic expression systems to achieve high levels of VT antigen production.

The sequence of the toxin gene has been determined (*see e.g.*, M.P. Jackson *et al.*, "Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," 44:109 [1987]). The coding regions of the A and B subunits of VT-1 are listed in SEQ ID NOS:1 and 3, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-1 toxin are listed in SEQ ID NOS:2 and 4, respectively. The coding regions of the A and B subunits of VT-2 are listed in SEQ ID NOS:5 and 7, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-2 toxin are listed in SEQ ID NOS:6 and 8, respectively. In addition, SEQ ID NOS:9 and 10 list the sequences which direct the expression of a poly-cistronic RNA capable of directing the translation of both the A and B subunits from the VT-1 and VT-2 genes, respectively.

In choosing a strategy for recombinant VT antigen production, there are three primary technical factors to consider. First, the appropriate VT antigen components representing the spectrum of toxin epitopes encountered in nature must be utilized. Second, the protein antigens must be expressed at sufficient levels and purity to enable immunization and large-scale antibody purification. Third, the neutralizing epitopes must be preserved in the immunogen and immunoabsorbant. Approaches that offer the greatest promise for high level expression of periplasmically localized, native, affinity-tagged proteins were developed. Figure 10 shows the fusion constructs of VT components and affinity tags.

A. Expression of affinity-tagged C-terminal constructs.

The VT1 and VT2 A and B subunits (SEQ ID NOS:1, 3, 5 and 7) are cloned into the pET-23b vector (Novagen). This vector is designed to allow expression of native proteins containing C-terminal poly-His tags. The vector utilizes a strong T7 polymerase promoter to drive high level expression of target proteins. The methionine initiation codon is engineered to contain a unique *Nde*I restriction enzyme site (CATATG). The VT1 and VT2 genes are engineered to convert the signal sequence methionine codon into a *Nde*I site utilizing PCR

mutagenesis. PCR primers were designed which contain the sequence GCCAT fused to the first 20–24 bases of the genes (starting at the ATG start codon of the signal tag: SEQ ID NOS:12-19, see Table below). Upon PCR amplification, the 5' start codon of each gene is converted to an *Nde*I site, compatible with the pET-23 vector-encoded *Nde*I site, allowing cloning of the amplified genes into the vector without the addition of vector-encoded amino acids.

Primers containing the C-terminal 7 codons of each gene (21 bases) fused to the sequence <u>CTCGAG</u>CC were synthesized, in order to add a C-terminal poly-His tag to each gene. The underlined bases are an *Xho*I site, that is compatible with the *Xho*I site of the pET-23 vector. These primers precisely delete the native stop codons, and when cloned into the pET-23 vector, add a C-terminal extension of "LeuGluHisHisHisHisHisHis" (SEQ ID NO: 11). The following table lists the primer pairs are utilized to create PCR fragments containing the A and B subunits derived from VT-1 and VT-2 toxin genes suitable for insertion into the pET-23b vector.

Table 9

Primers

Toxin Gene and Subunit	N-terminal Primer	C-terminal Primer
VT-1 Subunit A	SEQ ID NO:12	SEQ ID NO:13
VT-1 Subunit B	SEQ ID NO:14	SEQ ID NO:15
VT-2 Subunit A	SEQ ID NO:16	SEQ ID NO:17
VT-2 Subunit B	SEQ ID NO:18	SEQ ID NO:19
VT-1 Subunits A and B	SEQ ID NO:12	SEQ ID NO:15
VT-2 Subunits A and B	SEQ ID NO:16	SEQ ID NO:19

Thus, utilizing PCR amplification with the above modified N- and C-terminal primers, the A and B subunits of VT1 and VT2 are expressed as proteins containing an 8 amino acid C-terminal extension bearing an poly-histidine affinity tag. The amino acid sequence of the histidine-tagged VT-1 A subunit produced by expression from the pET-23b vector is listed in SEQ ID NO:21 (the associated DNA sequence is listed in SEQ ID NO:20); the amino acid sequence of the histidine-tagged VT-1 B subunit is listed in SEQ ID NO:23 (the associated

DNA sequence is listed in SEQ ID NO:22); the amino acid sequence of the histidine-tagged VT-2 A subunit is listed in SEQ ID NO:25 (the associated DNA sequence is listed in SEQ ID NO:24); the amino acid sequence of the histidine-tagged VT-2 B subunit is listed in SEQ ID NO:27 (the associated DNA sequence is listed in SEQ ID NO:26).

Both subunits may be expressed from a single expression constructs by utilizing SEQ ID NOS:12 and 15 to prime synthesis of the VT-1 toxin gene and SEQ ID NOS:16 and 19 to prime synthesis of the VT-2 toxin gene. The resulting PCR products are cleaved with *Nde*I and *Xho*I, as described for the cloning of the subunit genes into the pET-23b vector. Expression of the A and B subunits from such an expression vector, results in the expression of a native A subunit and a his-tagged B subunit. As the A and B subunits assemble into a complex, the presence of the his-tag on only the B subunit is sufficient to allow purification of the holotoxin on metal chelate columns as described below.

The proofreading *Pfu* polymerase (Stratagene) is utilized for PCR amplification to reduce the error rate during amplification. Genomic DNA from an *E. coli* O157:H7 strain is utilized as template DNA. Following the PCR, the amplification products are digested with *Nde*I and *Xho*I and cloned into the pCR-Script SK cloning vehicle (Stratagene) to permit DNA sequence analysis of the amplified products. The DNA sequence analysis is performed to ensure that no base changes are introduced during amplification. Once the desired clones are identified by DNA sequencing, the inserts are then excised utilizing *Nde*I and *Xho*I, and cloned into a similarly cut pET-23b vector to create the expression constructs. According to the published sequences, neither the VT1 nor VT2 genes contain either of these restriction sites.

The poly-His-tagged proteins produced by expression of the VT-1 and VT-2 gene sequences in the pET-23b constructs are then purified by IMAC. This method uses metal-chelate affinity chromatography to purify native or denatured proteins which have histidine tails (*see e.g.*, K. J. Petty, "Metal-Chelate Affinity Chromatography," *in* Current Protocols in Molecular Biology, Supplement 24, Unit 10.11B [1993]).

B. Expression of Toxin Containing N-terminal Affinity Tags

Two expression systems, pMal-p2 and pFLAG-1 are utilized to attach an N-terminal affinity tag to the A subunits from the VT-1 and VT-2 toxins.

MBP-tagged constructs. To construct A chains containing the maltose binding protein (MBP) at the N-terminus of the A subunit, PCR amplified gene products are cloned into the

pMal-p2 vector (New England Biolabs) as C-terminal fusions to a periplasmically-secreted version of the MBP. The MBP selectively binds to amylose resins and serves as an affinity tag on the MBP/A subunit fusion protein. The pMal-p2 vector contains an engineered factor Xa cleavage site, which permits the removal of the affinity tag (*i.e.*, MBP) from the fusion protein after purification.

The MBP/A subunit fusions are generated as follows. The VT1 and VT2 A subunits are PCR-amplified utilizing the following DNA primers. SEQ ID NOS:28-31: SEQ ID NOS:28 and 29 comprise the 5' and 3' primers, respectively, for the amplification of the VT1 A subunit; SEQ ID NOS:30 and 31 comprise the 5' and 3' primers, respectively, for the amplification of the VT2 A subunit. In both cases, the 5' or N-terminal primer contains the sequence CGGAATTC fused to the first codon of the mature polypeptide (rather than the start of the signal peptide, since the MBP signal peptide is utilized). These 5' primers contain an engineered *Eco*RI site that is not contained internally in either gene, that is compatible with the *Eco*RI site of the pMal-p2 vector. The 3' or C-terminal primers incorporate an *Xho*I site as described above for the generation of the His-tagged toxins, but in this case, the 3' primer is designed to include the natural termination codon of the A subunits.

The genes are amplified, cloned into pCR-Script SK, and sequenced as described above. The inserts are then excised with *Eco*RI and *Xho*I, and cloned into *Eco*RI/*Sal*I-cleaved pMal-p2 vector (*Sal*I and *Xho*I sites are compatible). This construct allows expression and secretion of the VT1 and VT2 A subunit genes as C-terminal fusions with MBP. The amino acid sequence of the MBP/VT-1A fusion protein is listed in SEQ ID NO:33 (the associated DNA sequence is listed in SEQ ID NO:32). The amino acid sequence of the MBP/VT-2A fusion protein is listed in SEQ ID NO:35 (the associated DNA sequence is listed in SEQ ID NO:34).

The resulting fusion proteins are then affinity purified on an amylose column and the bound fusion protein is eluted under mild conditions by competition with maltose. The MBP N-terminal-tagged A subunits are cleaved with factor Xa and the MBP is removed by chromatography on an amylose column. The resulting A subunits which contain a 4 amino acid N-terminal extension are then used as immunogens.

Flag tag constructs. In an alternative embodiment, the VT1 and VT2 A subunit genes are engineered to contain the "flag tag" through the use of the pFLAG-1 vector system. The flag tag is located between the *Omp*A secretion signal sequence and the authentic N-

terminus of the target protein in the pFlag-1 vector. To construct N-terminal flag-tagged A chains, the *EcoRI/XhoI* A subunit PCR fragments (generated as described above for the MBP fusion proteins) are cloned into identically cleaved pFlag-1 vector (Eastman-Kodak), to produce an expression construct utilizing the *Omp*A signal peptide for secretion of A subunit fusion proteins containing the flag peptide at the N-terminus. After secretion, the periplasmic protein contains the N-terminal 8 amino acid flag tag, followed by 4 vector-encoded amino acids fused to the recombinant A subunit. The amino acid sequence of the flag tag/VT-1 A subunit fusion protein is listed in SEQ ID NO:37 (the associated DNA sequence is listed in SEQ ID NO:36). The amino acid sequence of the flag tag/VT-2 A subunit fusion protein is listed in SEQ ID NO:39 (the associated DNA sequence is listed in SEQ ID NO:38).

The flag tag fusion proteins are then purified by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (Antiflag M1: Eastman-Kodak). Mild elution of purified protein is achieved by chelating the calcium in the column buffer with ethylenediamine tetraacetic acid (EDTA).

C. **Evaluation of fusion construct expression.**

The fusion constructs described above are expressed in *E. coli* strain BL21, or T7 polymerase-containing derivatives [*e.g.*, BL21(DE3), BL21(DE3) pLysS, BL21(DE3)pLysE] (Novagen) for pET plasmids, and periplasmically-secreted recombinant protein purified by affinity chromatography. Recombinant proteins are analyzed for correct conformation by testing the following parameters:

a) It is believed that the B subunit must associate into pentamers to be conformationally correct. This is assessed by reducing and native SDS-PAGE analyses of native and chemically-cross-linked proteins and sizing HPLC;

b) It is believed that a properly folded A subunit is expected to retain its native enzymatic activity. This is tested by its capacity to inhibit protein synthesis in an *in vitro* toxicity assay;

c) It is believed that *in vitro* toxicity of assembled recombinant holotoxin is compared to commercially available holotoxins to determine whether recombinant A and B subunits can assemble into functional holotoxin. The

purified N-terminal-tagged A subunits (after cleavage and purification from MBP or untreated flag-tagged proteins) are combined *in vitro* with the corresponding B chains, and their toxicity evaluated utilizing a quantitative microtiter cytotoxicity assay, such as that described by M.K. Gentry and M. Dalrymple, "Quantitative Microtiter Cytotoxicity Assay for *Shigella* Toxin," J. Clin. Microbiol., 12:361-366 (1980).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: OPHIDIAN PHARMACEUTICALS, INC.

(ii) TITLE OF INVENTION: TREATMENT FOR VEROTOXIN-PRODUCING E. COLI

(iii) NUMBER OF SEQUENCES: 39

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 945 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..945

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT      48
Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
  1               5                  10                  15

TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA      96
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
             20                  25                  30

AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT     144
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
         35                  40                  45

CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT     192
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
     50                  55                  60
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AGT GGC TCA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT      240
Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
 65                  70                  75                  80

GCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT      288
Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
                 85                  90                  95

AAT TTA TAT GTG ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT      336
Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
            100                 105                 110

CGC TTT GCT GAT TTT TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT      384
Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
        115                 120                 125

ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG      432
Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
    130                 135                 140

ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT      480
Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
145                 150                 155                 160

TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG      528
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
                165                 170                 175

GCA AGA GCG ATG TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT      576
Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
            180                 185                 190

TTT CGG CAA ATA CAG AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT      624
Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
        195                 200                 205

GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC      672
Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
    210                 215                 220

TGG GGA AGG TTG AGT AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT      720
Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
225                 230                 235                 240

GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA      768
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
                245                 250                 255

AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC      816
Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
            260                 265                 270

AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA      864
Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
        275                 280                 285

GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG      912
Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
    290                 295                 300

GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT                          945
Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 315 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
  1               5                  10                  15

Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
             20                  25                  30

Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
         35                  40                  45

Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
     50                  55                  60

Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
 65                  70                  75                  80

Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
                 85                  90                  95

Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
            100                 105                 110

Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
        115                 120                 125

Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
    130                 135                 140

Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
145                 150                 155                 160

Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
                165                 170                 175

Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
            180                 185                 190

Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
        195                 200                 205

Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
    210                 215                 220

Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
225                 230                 235                 240

Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
                245                 250                 255

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
            260                 265                 270

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
        275                 280                 285

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
    290                 295                 300

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..267

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA        48
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
  1               5                  10                  15

AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA        96
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
             20                  25                  30

AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA       144
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
         35                  40                  45

TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT CTC AGT GCG CAA       192
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
     50                  55                  60

ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA       240
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
 65                  70                  75                  80

GGG GGA TTC AGC GAA GTT ATT TTT CGT                                   267
Gly Gly Phe Ser Glu Val Ile Phe Arg
                 85
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
  1               5                  10                  15

Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
             20                  25                  30

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
         35                  40                  45

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
     50                  55                  60

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
 65                  70                  75                  80

Gly Gly Phe Ser Glu Val Ile Phe Arg
                 85
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 954 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..954

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT       48
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
 1               5                  10                  15

TCT TCG GTA TCC TAT TCC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA       96
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
            20                  25                  30

CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC      144
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
        35                  40                  45

CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC      192
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
    50                  55                  60

CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC      240
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
65                  70                  75                  80

TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT      288
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
                85                  90                  95

TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT      336
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
            100                 105                 110

TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC      384
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
        115                 120                 125

ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG      432
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
    130                 135                 140

GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT      480
Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145                 150                 155                 160

CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC      528
Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
                165                 170                 175

AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC      576
Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
            180                 185                 190

AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT      624
Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
        195                 200                 205

GTG TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG      672
Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
    210                 215                 220
```

```
CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA      720
Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225                 230                 235                 240

GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG      768
Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
            245                 250                 255

GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC      816
Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
        260                 265                 270

GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT      864
Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
    275                 280                 285

GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG      912
Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
    290                 295                 300

TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA              954
Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 318 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
  1               5                  10                  15

Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
            20                  25                  30

Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
        35                  40                  45

Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
    50                  55                  60

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
65                  70                  75                  80

Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
                85                  90                  95

Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
            100                 105                 110

Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
        115                 120                 125

Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
    130                 135                 140

Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145                 150                 155                 160

Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
                165                 170                 175

Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
            180                 185                 190
```

```
Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
        195                 200                 205

Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
    210                 215                 220

Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225                 230                 235                     240

Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
                245                 250                 255

Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
            260                 265                 270

Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
        275                 280                 285

Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
    290                 295                 300

Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..267

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT      48
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
  1               5                  10                  15

GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT      96
Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
             20                  25                  30

AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG     144
Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
         35                  40                  45

ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA     192
Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
     50                  55                  60

GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA     240
Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65                  70                  75                  80

TTT GCT GAA GTG CAG TTT AAT AAT GAC                                 267
Phe Ala Glu Val Gln Phe Asn Asn Asp
                 85
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
  1               5                  10                  15

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
             20                  25                  30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
         35                  40                  45

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
     50                  55                  60

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65                  70                  75                  80

Phe Ala Glu Val Gln Phe Asn Asn Asp
                 85
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1241 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATGAAAATAA TTATTTTTAG AGTGCTAACT TTTTTCTTTG TTATCTTTTC AGTTAATGTG    60
GTGGCGAAGG AATTTACCTT AGACTTCTCG ACTGCAAAGA CGTATGTAGA TTCGCTGAAT   120
GTCATTCGCT CTGCAATAGG TACTCCATTA CAGACTATTT CATCAGGAGG TACGTCTTTA   180
CTGATGATTG ATAGTGGCTC AGGGGATAAT TTGTTTGCAG TTGATGTCAG AGGGATAGAT   240
GCAGAGGAAG GGCGGTTTAA TAATCTACGG CTTATTGTTG AACGAAATAA TTTATATGTG   300
ACAGGATTTG TTAACAGGAC AAATAATGTT TTTTATCGCT TTGCTGATTT TTCACATGTT   360
ACCTTTCCAG GTACAACAGC GGTTACATTG TCTGGTGACA GTAGCTATAC CACGTTACAG   420
CGTGTTGCAG GGATCAGTCG TACGGGGATG CAGATAAATC GCCATTCGTT GACTACTTCT   480
TATCTGGATT TAATGTCGCA TAGTGGAACC TCACTGACGC AGTCTGTGGC AAGAGCGATG   540
TTACGGTTTG TTACTGTGAC AGCTGAAGCT TTACGTTTTC GGCAAATACA GAGGGGATTT   600
CGTACAACAC TGGATGATCT CAGTGGGCGT TCTTATGTAA TGACTGCTGA AGATGTTGAT   660
CTTACATTGA ACTGGGGAAG GTTGAGTAGC GTCCTGCCTG ACTATCATGG ACAAGACTCT   720
GTTCGTGTAG GAAGAATTTC TTTTGGAAGC ATTAATGCAA TTCTGGGAAG CGTGGCATTA   780
ATACTGAATT GTCATCATCA TGCATCGCGA GTTGCCAGAA TGGCATCTGA TGAGTTTCCT   840
TCTATGTGTC CGGCAGATGG AAGAGTCCGT GGGATTACGC ACAATAAAAT ATTGTGGGAT   900
```

TCATCCACTC TGGGGGCAAT TCTGATGCGC AGAACTATTA GCAGTTGAAC AGGGGGTAAA 960

TAAAGGAGTT AAGCATGAAA AAAACATTAT TAATAGCTGC ATCGCTTTCA TTTTTTTCAG 1020

CAAGTGCGCT GGCGACGCCT GATTGTGTAA CTGGAAAGGT GGAGTATACA AAATATAATG 1080

ATGACGATAC CTTTACAGTT AAAGTGGGTG ATAAAGAATT ATTTACCAAC AGATGGAATC 1140

TTCAGTCTCT TCTTCTCAGT GCGCAAATTA CGGGGATGAC TGTAACCATT AAAACTAATG 1200

CCTGTCATAA TGGAGGGGGA TTCAGCGAAG TTATTTTTCG T 1241

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1235 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGAAGTGTA TATTATTTAA ATGGGTACTG TGCCTGTTAC TGGGTTTTTC TTCGGTATCC 60

TATTCCCGGG AGTTTACGAT AGACTTTTCG ACCCAACAAA GTTATGTCTC TTCGTTAAAT 120

AGTATACGGA CAGAGATATC GACCCCTCTT GAACATATAT CTCAGGGGAC CACATCGGTG 180

TCTGTTATTA ACCACACCCA CGGCAGTTAT TTTGCTGTGG ATATACGAGG GCTTGATGTC 240

TATCAGGCGC GTTTTGACCA TCTTCGTCTG ATTATTGAGC AAAATAATTT ATATGTGGCA 300

GGGTTCGTTA ATACGGCAAC AAATACTTTC TACCGTTTTT CAGATTTTAC ACATATATCA 360

GTGCCCGGTG TGACAACGGT TTCCATGACA ACGGACAGCA GTTATACCAC TCTGCAACGT 420

GTCGCAGCGC TGGAACGTTC CGGAATGCAA ATCAGTCGTC ACTCACTGGT TTCATCATAT 480

CTGGCGTTAA TGGAGTTCAG TGGTAATACA ATGACCAGAG ATGCATCCAG AGCAGTTCTG 540

CGTTTTGTCA CTGTCACAGC AGAAGCCTTA CGCTTCAGGC AGATACAGAG AGAATTTCGT 600

CAGGCACTGT CTGAAACTGC TCCTGTGTAT ACGATGACGC CGGGAGACGT GGACCTCACT 660

CTGAACTGGG GGCGAATCAG CAATGTGCTT CCGGAGTATC GGGGAGAGGA TGGTGTCAGA 720

GTGGGGAGAA TATCCTTTAA TAATATATCA GCGATACTGG GGACTGTGGC CGTTATACTG 780

AATTGCCATC ATCAGGGGGC GCGTTCTGTT CGCGCCGTGA ATGAAGAGAG TCAACCAGAA 840

TGTCAGATAA CTGGCGACAG GCCTGTTATA AAAATAAACA ATACATTATG GGAAAGTAAT 900

ACAGCTGCAG CGTTTCTGAA CAGAAAGTCA CAGTTTTTAT ATACAACGGG TAAATAAAGG 960

AGTTAAGCAT GAAGAAGATG TTTATGGCGG TTTTATTTGC ATTAGCTTCT GTTAATGCAA 1020

TGGCGGCGGA TTGTGCTAAA GGTAAAATTG AGTTTTCCAA GTATAATGAG GATGACACAT 1080

TTACAGTGAA GGTTGACGGG AAAGAATACT GGACCAGTCG CTGGAATCTG CAACCGTTAC 1140

TGCAAAGTGC TCAGTTGACA GGAATGACTG TCACAATCAA ATCCAGTACC TGTGAATCAG 1200

GCTCCGGATT TGCTGAAGTG CAGTTTAATA ATGAC 1235

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Glu His His His His His His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCATATGAA AATAATTATT TTTAGAGTG 29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCTCGAGAC TGCTAATAGT TCTGCGCAT 29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCATATGAA AAAAACATTA TTAATAGC 28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGCTCGAGAC GAAAAATAAC TTCGCTGAA 29

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCATATGAA GTGTATATTA TTTAAATGG 29

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCTCGAGTT TACCCGTTGT ATATAAAAAC 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCATATGAA GAAGATGTTT ATGGCG 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCTCGAGGT CATTATTAAA CTGCACTTC 29

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 969 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..969

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT 48

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
1 5 10 15

TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA 96
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
20 25 30

AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT 144
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
35 40 45

CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT 192
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
50 55 60

AGT GGC TCA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT 240
Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
65 70 75 80

GCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT 288
Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
85 90 95

AAT TTA TAT GTG ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT 336
Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
100 105 110

CGC TTT GCT GAT TTT TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT 384
Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
115 120 125

ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG 432
Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
130 135 140

ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT 480
Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
145 150 155 160

TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG 528
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
165 170 175

GCA AGA GCG ATG TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT 576
Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
180 185 190

TTT CGG CAA ATA CAG AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT 624
Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
195 200 205

GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC 672
Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
210 215 220

TGG GGA AGG TTG AGT AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT 720
Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
225 230 235 240

GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA 768
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
245 250 255

AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC 816
Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
260 265 270

AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA 864
Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
275 280 285

```
GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG        912
Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
    290                 295                 300

GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT CTC GAG CAC CAC CAC        960
Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His
305                 310                 315                 320

CAC CAC CAC                                                            969
His His His
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 323 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
 1               5                  10                  15

Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
            20                  25                  30

Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
        35                  40                  45

Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
    50                  55                  60

Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
65                  70                  75                  80

Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
                85                  90                  95

Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
            100                 105                 110

Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
        115                 120                 125

Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
    130                 135                 140

Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
145                 150                 155                 160

Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
                165                 170                 175

Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
            180                 185                 190

Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
        195                 200                 205

Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
    210                 215                 220

Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
225                 230                 235                 240

Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
                245                 250                 255
```

```
Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
            260                 265                 270

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
        275                 280                 285

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
    290                 295                 300

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His
305                 310                 315                 320

His His His
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 294 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA       48
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
  1               5                  10                  15

AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA       96
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
            20                  25                  30

AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA      144
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
        35                  40                  45

TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT CTC AGT GCG CAA      192
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
    50                  55                  60

ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA      240
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
 65                  70                  75                  80

GGG GGA TTC AGC GAA GTT ATT TTT CGT CTC GAG CAC CAC CAC CAC CAC      288
Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His
                85                  90                  95

CAC TG                                                               294
His
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
```

```
1               5                   10                  15

Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
            20                  25                  30

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
        35                  40                  45

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
    50                  55                  60

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
65                  70                  75                  80

Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His
                85                  90                  95

His
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 981 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..981

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT       48
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
1               5                   10                  15

TCT TCG GTA TCC TAT TCC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA       96
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
            20                  25                  30

CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC      144
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
        35                  40                  45

CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC      192
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
    50                  55                  60

CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC      240
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
65                  70                  75                  80

TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT      288
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
                85                  90                  95

TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT      336
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
            100                 105                 110

TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC      384
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
        115                 120                 125

ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG      432
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
    130                 135                 140
```

```
GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT      480
Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145                 150                 155                 160

CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC      528
Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
                165                 170                 175

AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC      576
Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
            180                 185                 190

AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT      624
Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
        195                 200                 205

GTG TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG      672
Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
    210                 215                 220

CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA      720
Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225                 230                 235                 240

GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG      768
Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
                245                 250                 255

GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC      816
Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
            260                 265                 270

GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT      864
Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
        275                 280                 285

GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG      912
Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
    290                 295                 300

TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA CTC GAG      960
Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu
305                 310                 315                 320

CAC CAC CAC CAC CAC CAC TG                                           981
His His His His His His
                325
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 326 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
  1               5                  10                  15

Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
            20                  25                  30

Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
        35                  40                  45

Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
    50                  55                  60
```

```
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
 65                 70                  75                  80

Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
                85                  90                  95

Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
            100                 105                 110

Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
        115                 120                 125

Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
    130                 135                 140

Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145                 150                 155                 160

Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
                165                 170                 175

Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
            180                 185                 190

Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
        195                 200                 205

Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
    210                 215                 220

Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225                 230                 235                 240

Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
                245                 250                 255

Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
            260                 265                 270

Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
        275                 280                 285

Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
    290                 295                 300

Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu
305                 310                 315                 320

His His His His His His
                325
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 294 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT       48
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
  1               5                  10                  15
```

GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT 96
Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
20 25 30

AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG 144
Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
35 40 45

ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA 192
Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
50 55 60

GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA 240
Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
65 70 75 80

TTT GCT GAA GTG CAG TTT AAT AAT GAC CTC GAG CAC CAC CAC CAC CAC 288
Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His
85 90 95

CAC TG 294
His

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
1 5 10 15

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
20 25 30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
35 40 45

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
50 55 60

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
65 70 75 80

Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His
85 90 95

His

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGAATTCAA GGAATTTACC TTAGACTTCT CG 32

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGCTCGAGTC AACTGCTAAT AGTTCTGC 28

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGGAATTCCG GGAGTTTACG ATAGACTTTT CG 32

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGCTCGAGTT ATTTACCCGT TGTATATAA 29

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2127

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

```
ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG      48
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
  1               5                  10                  15

ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA      96
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
             20                  25                  30

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA     144
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
         35                  40                  45

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG     192
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
```

```
     50                  55                  60

CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC      240
His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
 65                  70                  75                  80

GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC      288
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                 85                  90                  95

GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG      336
Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG      384
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC      432
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
    130                 135                 140

AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG      480
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145                 150                 155                 160

CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC      528
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
                165                 170                 175

CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT      576
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
            180                 185                 190

TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC      624
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
        195                 200                 205

GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG      672
Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
    210                 215                 220

ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA      720
Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225                 230                 235                 240

GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG      768
Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
                245                 250                 255

GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA      816
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG      864
Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG      912
Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300
```

```
TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT      960
Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                 320

AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG     1008
Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA     1056
Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
            340                 345                 350

GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC     1104
Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT     1152
Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT     1200
Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385                 390                 395                 400

AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC AAG     1248
Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys
                405                 410                 415

GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT TCG CTG     1296
Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu
            420                 425                 430

AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT TCA TCA     1344
Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser
        435                 440                 445

GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT AAT TTG     1392
Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu
    450                 455                 460

TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG TTT AAT     1440
Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn
465                 470                 475                 480

AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA GGA TTT     1488
Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe
                485                 490                 495

GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT TCA CAT     1536
Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His
            500                 505                 510

GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC AGT AGC     1584
Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser
        515                 520                 525

TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG ATG CAG     1632
Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln
    530                 535                 540

ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG TCG CAT     1680
Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His
545                 550                 555                 560

AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA CGG TTT     1728
Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe
                565                 570                 575

GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG AGG GGA     1776
Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly
```

580 585 590

TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA ATG ACT 1824
Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr
595 600 605

GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT AGC GTC 1872
Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val
610 615 620

CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA ATT TCT 1920
Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser
625 630 635 640

TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA CTG AAT 1968
Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn
645 650 655

TGT CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT GAG TTT 2016
Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe
660 665 670

CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG CAC AAT 2064
Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn
675 680 685

AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG CGC AGA 2112
Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg
690 695 700

ACT ATT AGC AGT TG 2127
Thr Ile Ser Ser
705

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 708 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
1 5 10 15

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
20 25 30

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
35 40 45

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
50 55 60

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
65 70 75 80

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
85 90 95

Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
100 105 110

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
115 120 125

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn

130 135 140

Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145 150 155 160

Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
165 170 175

Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
180 185 190

Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
195 200 205

Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
210 215 220

Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225 230 235 240

Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
245 250 255

Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
260 265 270

Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
275 280 285

Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
290 295 300

Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305 310 315 320

Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
325 330 335

Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
340 345 350

Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
355 360 365

Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
370 375 380

Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385 390 395 400

Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys
405 410 415

Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu
420 425 430

Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser
435 440 445

Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu
450 455 460

Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn
465 470 475 480

Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe
485 490 495

Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His
500 505 510

```
Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser
        515                 520                 525

Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln
    530                 535                 540

Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His
545                 550                 555                     560

Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe
                565                 570                 575

Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly
            580                 585                 590

Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr
        595                 600                 605

Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val
    610                 615                 620

Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser
625                 630                 635                     640

Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn
                645                 650                 655

Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe
            660                 665                 670

Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn
        675                 680                 685

Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg
    690                 695                 700

Thr Ile Ser Ser
705
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2136 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2136

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG      48
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
  1               5                  10                  15

ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA      96
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
             20                  25                  30

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA     144
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
         35                  40                  45

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG     192
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
     50                  55                  60
```

```
CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC       240
His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
 65                  70                  75                  80

GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC       288
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                 85                  90                  95

GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG       336
Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG       384
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC       432
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
    130                 135                 140

AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG       480
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145                 150                 155                 160

CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC       528
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
                165                 170                 175

CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT       576
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
            180                 185                 190

TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC       624
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
        195                 200                 205

GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG       672
Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
    210                 215                 220

ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA       720
Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225                 230                 235                 240

GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG       768
Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
                245                 250                 255

GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA       816
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG       864
Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG       912
Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300

TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT       960
Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                 320

AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG      1008
Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA      1056
Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
```

```
            340                 345                 350

GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC      1104
Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT      1152
Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT      1200
Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385                 390                 395                     400

AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC CGG      1248
Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg
                405                 410                 415

GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT TCG TTA      1296
Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu
            420                 425                 430

AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA TCT CAG      1344
Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln
        435                 440                 445

GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT TAT TTT      1392
Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe
    450                 455                 460

GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT CAG GCG CGT TTT GAC CAT      1440
Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His
465                 470                 475                     480

CTT CGT CTG ATT ATT GAG CAA AAT AAT TTA TAT GTG GCA GGG TTC GTT      1488
Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val
                485                 490                 495

AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA CAT ATA      1536
Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile
            500                 505                 510

TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC AGT TAT      1584
Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr
        515                 520                 525

ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG CAA ATC      1632
Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile
    530                 535                 540

AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG TTC AGT      1680
Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser
545                 550                 555                     560

GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT TTT GTC      1728
Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val
                565                 570                 575

ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA GAA TTT      1776
Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe
            580                 585                 590

CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG CCG GGA      1824
Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly
        595                 600                 605

GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG CTT CCG      1872
Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro
    610                 615                 620
```

```
GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC TTT AAT      1920
Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn
625                 630                 635                 640

AAT ATA TCA GCG ATA CTG GGG ACT GTG GCC GTT ATA CTG AAT TGC CAT      1968
Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His
                645                 650                 655

CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT CAA CCA      2016
His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser Gln Pro
            660                 665                 670

GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC AAT ACA      2064
Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr
        675                 680                 685

TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG TCA CAG      2112
Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln
    690                 695                 700

TTT TTA TAT ACA ACG GGT AAA TA                                       2136
Phe Leu Tyr Thr Thr Gly Lys
705                 710
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
  1               5                  10                  15

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
             20                  25                  30

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
         35                  40                  45

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
     50                  55                  60

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
 65                  70                  75                  80

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                 85                  90                  95

Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
    130                 135                 140

Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145                 150                 155                 160

Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
                165                 170                 175

Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
            180                 185                 190
```

```
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
        195                 200                 205

Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
    210                 215                 220

Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225                 230                 235                 240

Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
                245                 250                 255

Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300

Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                 320

Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
            340                 345                 350

Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385                 390                 395                 400

Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg
                405                 410                 415

Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu
            420                 425                 430

Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln
        435                 440                 445

Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe
    450                 455                 460

Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His
465                 470                 475                 480

Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val
                485                 490                 495

Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile
            500                 505                 510

Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr
        515                 520                 525

Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile
    530                 535                 540

Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser
545                 550                 555                 560

Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val
```

```
                565                 570                 575

Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe
            580                 585                 590

Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly
        595                 600                 605

Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro
    610                 615                 620

Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn
625                 630                 635                 640

Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His
                645                 650                 655

His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser Gln Pro
            660                 665                 670

Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr
        675                 680                 685

Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln
    690                 695                 700

Phe Leu Tyr Thr Thr Gly Lys
705                 710
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 981 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..981

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT      48
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
  1               5                  10                  15

ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG AAG CTT GAA      96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
             20                  25                  30

TTC AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT     144
Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp
         35                  40                  45

TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT     192
Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile
     50                  55                  60

TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT     240
Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp
 65                  70                  75                  80

AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG     288
Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg
                 85                  90                  95

TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA     336
```

```
Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr
            100                 105                 110

GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT      384
Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe
        115                 120                 125

TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC      432
Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp
    130                 135                 140

AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG      480
Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly
145                 150                 155                 160

ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG      528
Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met
                165                 170                 175

TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA      576
Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu
            180                 185                 190

CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG      624
Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln
        195                 200                 205

AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA      672
Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val
    210                 215                 220

ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT      720
Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser
225                 230                 235                 240

AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA      768
Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg
                245                 250                 255

ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA      816
Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile
            260                 265                 270

CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT      864
Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp
        275                 280                 285

GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG      912
Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr
    290                 295                 300

CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG      960
His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met
305                 310                 315                 320

CGC AGA ACT ATT AGC AGT TG                                           981
Arg Arg Thr Ile Ser Ser
                325
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 326 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
  1               5                  10                  15

Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
            20                  25                  30

Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp
        35                  40                  45

Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile
    50                  55                  60

Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp
 65                  70                  75                  80

Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg
                85                  90                  95

Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr
            100                 105                 110

Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe
        115                 120                 125

Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp
    130                 135                 140

Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly
145                 150                 155                 160

Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met
                165                 170                 175

Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu
            180                 185                 190

Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln
        195                 200                 205

Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val
    210                 215                 220

Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser
225                 230                 235                 240

Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg
                245                 250                 255

Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile
            260                 265                 270

Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp
        275                 280                 285

Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr
    290                 295                 300

His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met
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Arg Arg Thr Ile Ser Ser
                325
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(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 990 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

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  1               5                  10                  15

ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG AAG CTT GAA       96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
             20                  25                  30

TTC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT      144
Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser
         35                  40                  45

TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA      192
Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile
     50                  55                  60

TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT      240
Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser
 65                  70                  75                  80

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Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe
                 85                  90                  95

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Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly
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TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA      384
Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr
        115                 120                 125

CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC      432
His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser
    130                 135                 140

AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG      480
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145                 150                 155                 160

CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG      528
Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu
                165                 170                 175

TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT      576
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            180                 185                 190

TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA      624
Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg
        195                 200                 205

GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG      672
Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr
    210                 215                 220

CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG      720
Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val
225                 230                 235                 240

CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC      768
Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser
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                    245                 250                 255

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TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT        864
Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser
        275                 280                 285

CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC        912
Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn
    290                 295                 300

AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG        960
Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys
305                 310                 315                 320

TCA CAG TTT TTA TAT ACA ACG GGT AAA TA                                 990
Ser Gln Phe Leu Tyr Thr Thr Gly Lys
                325                 330
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(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

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Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
            20                  25                  30

Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser
        35                  40                  45

Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile
    50                  55                  60

Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser
65                  70                  75                  80

Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe
                85                  90                  95

Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly
            100                 105                 110

Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr
        115                 120                 125

His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser
    130                 135                 140

Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met
145                 150                 155                 160

Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu
                165                 170                 175

Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg
            180                 185                 190

Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg
```

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        195                 200                 205

Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr
    210                 215                 220

Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val
225                 230                 235                 240

Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser
                245                 250                 255

Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn
            260                 265                 270

Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser
        275                 280                 285

Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn
    290                 295                 300

Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys
305                 310                 315                 320

Ser Gln Phe Leu Tyr Thr Thr Gly Lys
                325
```

CLAIMS

What is claimed is:

1. A method of treatment comprising:

   a) providing:

      i) antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable. and

      ii) an intoxicated subject: and

   b) administering said antitoxin to said subject.

2. The method of Claim 1 wherein said *Escherichia coli* verotoxin is recombinant.

3. The method of Claim 1 wherein said antitoxin is an avian antitoxin.

4. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.

5. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

6. The method of Claim 1 wherein said subject is an adult.

7. The method of Claim 1 wherein said subject is a child.

8. The method of Claim 1 wherein said administering is parenteral.

9. The method of Claim 1 wherein said administering is oral.

10. A method of prophylactic treatment comprising:

a) providing:

i) an antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is parenterally administrable, and

ii) at least one subject is at risk of diarrheal disease: and

b) parenterally administering said antitoxin to said subject.

11. The method of Claim 10, wherein said subject is at risk of developing extra-intestinal complications of *Escherichia coli* infection.

12. The method of Claim 11, wherein said extra-intestinal complication is hemolytic uremic syndrome.

13. A composition comprising neutralizing antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amounts.

14. The composition of Claim 13 wherein said *Escherichia coli* verotoxin is a recombinant toxin.

15. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.

16. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

17. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

18. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1.

19. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2.

20. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

21. The composition of Claim 14 wherein said antitoxin is an avian antitoxin.

22. A method of treatment of enteric bacterial infections comprising:

a) providing:

i) an avian antitoxin directed against at least one verotoxin produced by *Escherichia coli* in an aqueous solution in therapeutic amount that is parenterally administrable, and

ii) at least one infected subject; and

b) parenterally administering said avian antitoxin to said subject.

23. The method of Claim 18 wherein said *Escherichia coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7, O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O163:H19; O165:NM; O165:19; and O165:H25

24. The method of Claim 22 wherein said antitoxin comprises antitoxin directed against at least one *Escherichia coli* verotoxin.

25. The method of Claim 22 wherein said antitoxin is cross-reactive with at least one *Escherichia coli* verotoxin.

26. The method of Claim 22 wherein said antitoxin is reactive against toxins produced by members of the genus *Shigella*.

27. The method of Claim 26, wherein said antitoxin is reactive against toxins produced by *Shigella dysenteriae*.

28. A method for detecting *Escherichia coli* verotoxin in a sample comprising:

a) providing:

i) a sample:

ii) an antitoxin raised against *Escherichia coli* verotoxin: and

iii) a reporter reagent capable of binding said antitoxin: and

b) adding said antitoxin to said sample so that said antitoxin binds to the Escherichia coli verotoxin in said sample.

29. The method of Claim 28, wherein said antitoxin is an avian antitoxin.

30. The method of Claim 28, further comprising the steps of:

c) washing said unbound antitoxin from said sample:

d) adding said reporter reagent to said sample so that said reporter reagent binds to said bound antitoxin:

e) washing said unbound reporter reagent from said sample: and

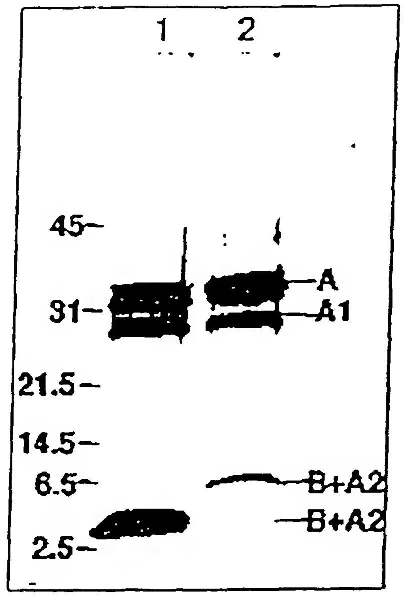
f) detecting said reporter reagent bound to said antitoxin bound to the *Escherichia coli* verotoxin so that the verotoxin is detected.

31. The method of Claim 30 wherein said detecting is selected from the group consisting of enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay.

32. The method of Claim 30 wherein said sample is a biological sample.

33. The method of Claim 30 wherein said sample is an environmental sample.

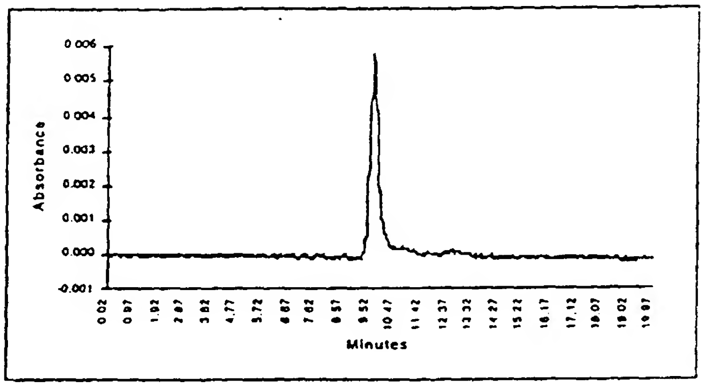
Figure 1.
SDS-PAGE of rVT1 and rVT2



rVT1 (Lane 1) and rVT2 (Lane 2). Positions of molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

Figure 2.

HPLC of rVT1



HPLC of rVT2

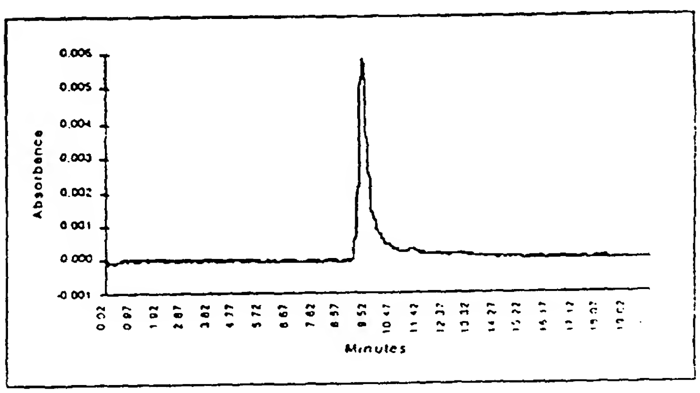


Figure 3.
rVT1 and rVT2 Toxicity in Vero Cell Culture

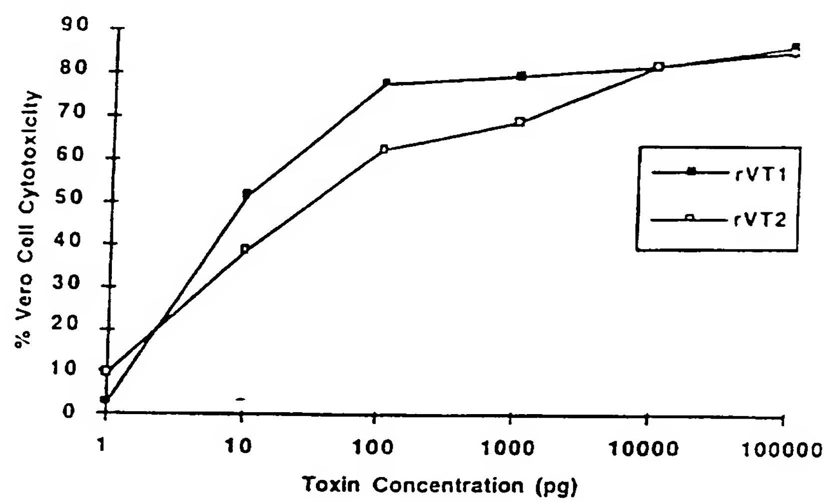


Figure 4.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT1

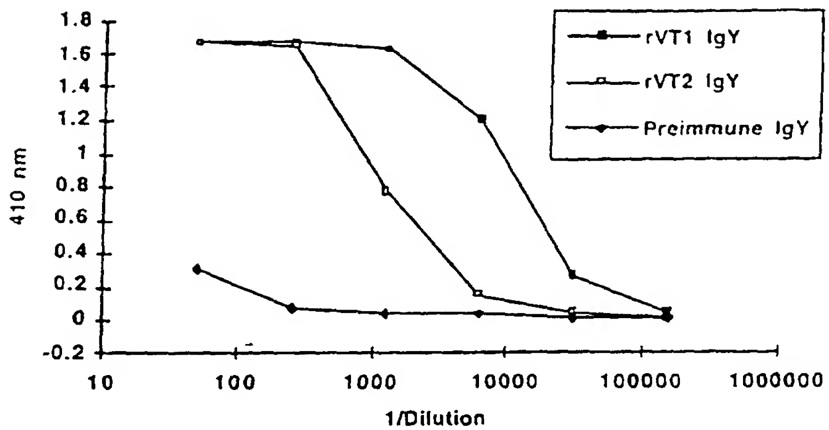


Figure 5.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT2

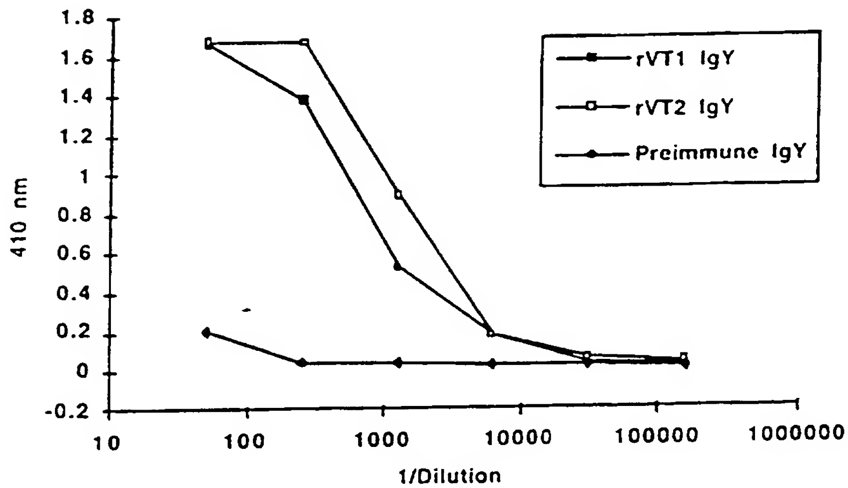
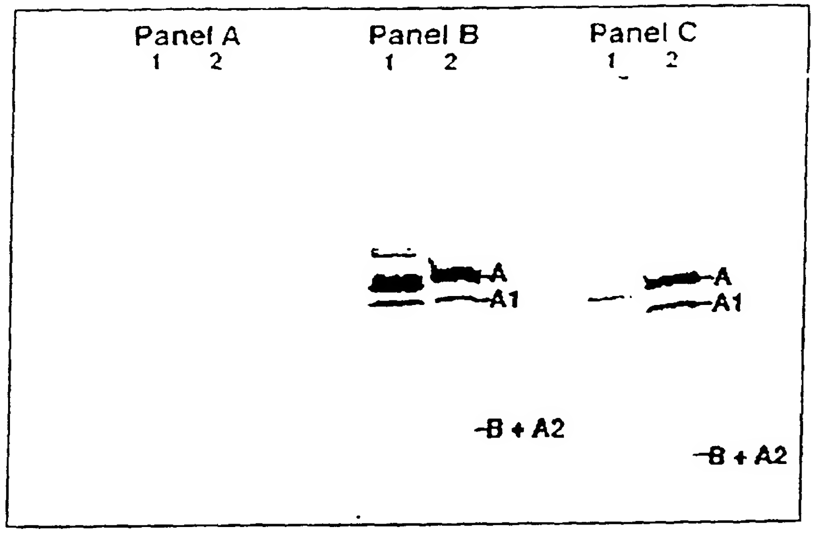


Figure 6.
Western Blot Reactivity of rVT1 and rVT2 Antibodies to rVT's



In this Figure, Panel A contains preimmune IgY, Panel B contains rVT1 IgY, and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 (2μg) and Lane 2 contains rVT2 (2 μg).

Figure 7.

Neutralization of rVT1 Cytotoxicity in Vero Cells

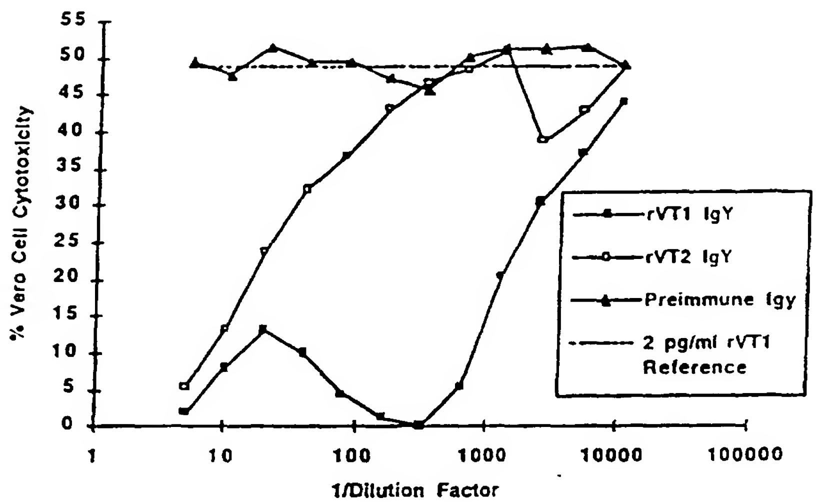


Figure 8.

Neutralization of rVT2 Cytotoxicity in Vero Cells

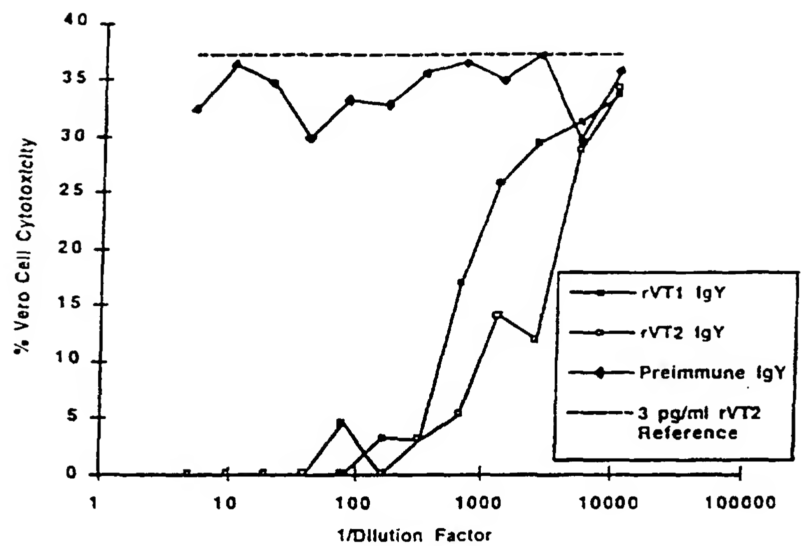
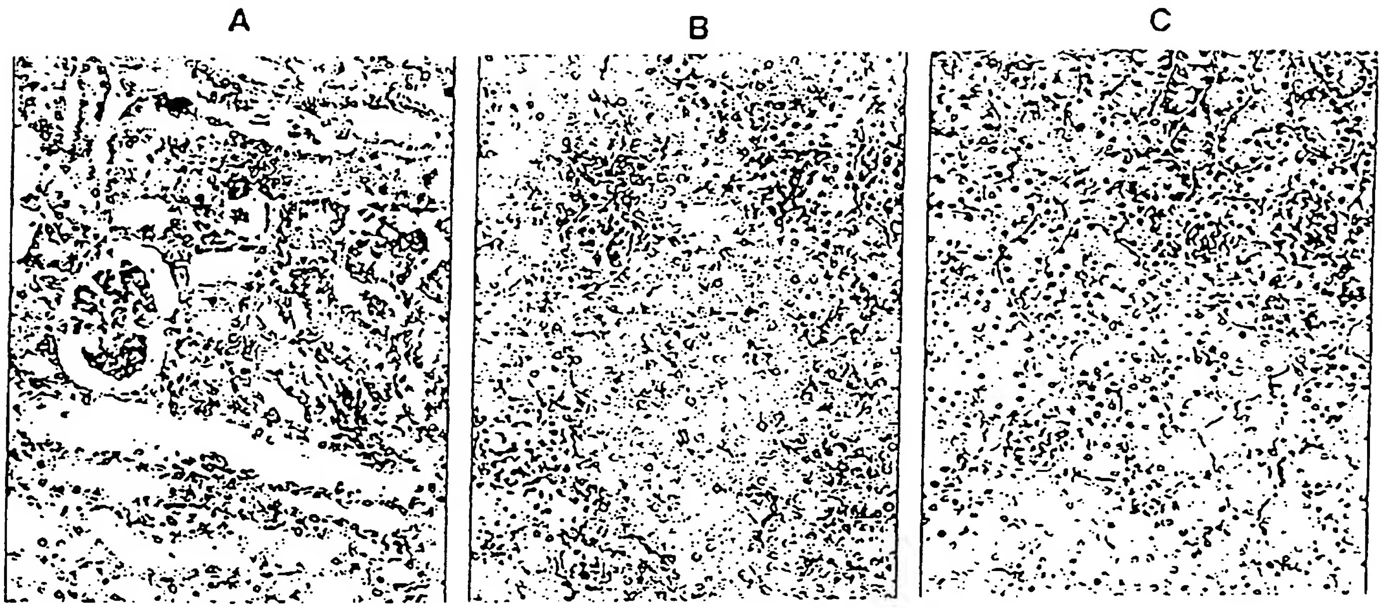


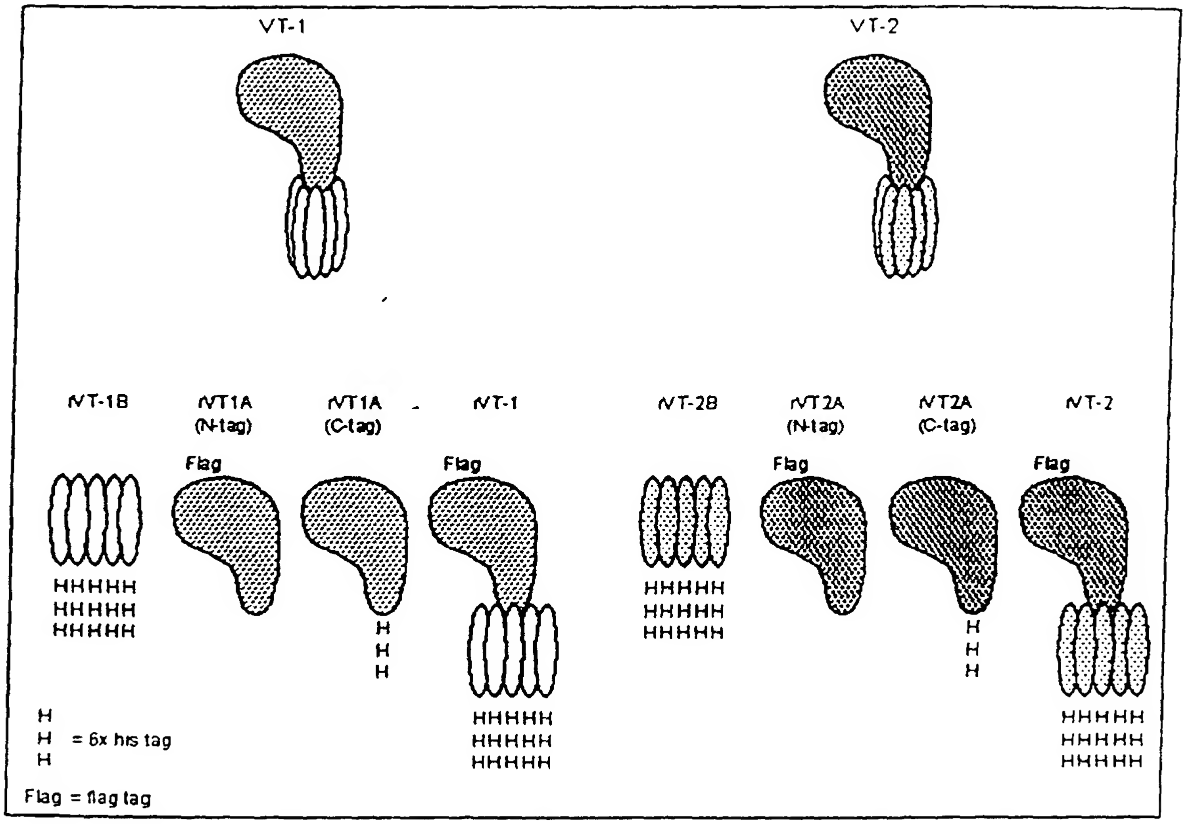
Figure 9.

Renal Sections from *E. coli* O157:H7-Infected Mice Treated with IgY



Representative kidney sections from mice treated with preimmune (Panel A), rVT1 (Panel B) or rVT2 (Panel C) IgY 4 hrs. after infection.

Figure 10.
Fusion Constructs of VT Components and Affinity Tags



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/04093

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/00, 39/02; G01N 33/537
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOYD et al. Serological Responses to the B Subunit of Shiga-Like Toxin 1 and Its Peptide Fragments Indicate that the B Subunit Is a Vaccine Candidate To Counter the Action of the Toxin. Infection and Immunity. March 1991, Vol. 59, No. 3, pages 750-757.	1-33
Y	US 5,326,559 A (MILLER) 05 July 1994, columns 4-7.	1-33
X --- Y	US 5,164,298 A (LINGWOOD et al) 17 November 1992, columns 10-13.	28, 30, 31, 32, 33 ---------- 1-27 and 29
Y	US 4,748,018 A (STOLLE et al) 31 May 1988, column 4, lines 25-55.	3, 21, 22, 29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 JULY 1996	27 AUG 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	RACHEL FREED
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,550,019 A (POLSON) 29 October 1985, column 4, lines 46-68.	3, 21, 22, 29
Y	US 5,204,097 A (ARNON et al) 20 April 1993, column 2, lines 1-16, column 3, lines 33-56 and column 5, lines 53-67.	2 and 14

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, MEDLINE, APS
search terms: verotoxin, verocytoxin, shiga, rvt1, rvt2, rslt1 or rslt2, vaccin? or treat?, recombinant

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHIA COLI

(57) Abstract

The present invention includes methods for generating neutralizing antitoxin directed against verotoxins. In particular, the antitoxin directed against these toxins is produced in avian species using soluble recombinant verotoxin proteins. This avian antitoxin is designed so as to be administrable in therapeutic amounts and may be in any form (i.e., as a solid or in aqueous solution). These antitoxins are useful in the treatment of humans and other animals intoxicated with at least one bacterial toxin, as well as for diagnostic assays to detect the presence of toxin in a sample.

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TREATMENT FOR VEROTOXIN-PRODUCING *ESCHERICHIA COLI*

FIELD OF THE INVENTION

The present invention relates to antitoxin therapy for humans and other animals, and diagnostic assays to detect toxins. Antitoxins which neutralize the pathologic effects of *Escherichia coli* toxins, such as verotoxin are provided.

BACKGROUND OF THE INVENTION

A. *Escherichia coli* as a Pathogenic Organism

Escherichia coli is the organism most commonly isolated in clinical microbiology laboratories, as it is usually present as normal flora in the intestines of humans and other animals. However, it is an important cause of intestinal, as well as extraintestinal infections. For example, in a 1984 survey of nosocomial infections in the United States, *E. coli* was associated with 30.7% of the urinary tract infections, 11.5% of the surgical wound infections, 6.4% of the lower respiratory tract infections, 10.5% of the primary bacteremia cases, 7.0% of the cutaneous infections, and 7.4% of the other infections (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae," *in* Manual of Clinical Microbiology, Balows *et al.*(eds), American Society for Microbiology, [1991], p. 365). Surveillance reports from England, Wales and Ireland for 1986 indicate that *E. coli* was responsible for 5,473 cases of bacteremia (including blood, bone marrow, spleen and heart specimens); of these, 568 were fatal. For spinal fluid specimens, there were 58 cases, with 10 fatalities (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae," *in* Manual of Clinical Microbiology, Balows *et al.*(eds), American Society for Microbiology, [1991], p. 366). There are no similar data for United States, as these are not reportable diseases in this country.

Studies in various countries have identified certain serotypes (based on both the O and H antigens) that are associated with the four major groups of *E. coli* recognized as enteric pathogens. Table 1 lists common serotypes included within these groups. The first group includes the classical enteropathogenic serotypes ("EPEC"); the next group includes those that produce heat-labile or heat-stable enterotoxins ("ETEC"); the third group includes the enteroinvasive strains ("EIEC") that mimic *Shigella* strains in their ability to invade and multiply within intestinal epithelial cells; and the fourth group includes strains and serotypes that cause hemorrhagic colitis or produce Shiga-like toxins (or verotoxins) ("VTEC" or "EHEC" [enterohemmorrhagic *E. coli*]).

Table 1.
Pathogenic *E. coli* Serotypes

Group	Associated Serotypes
Enterotoxigenic (ETEC)	O6:H16; O8:NM; O8:H9; O11:H27; O15:H11; O20:NM; O25:NM; O25:H42; O27:H7; O27:H20; O63:H12; O78:H11; O78:H12; O85:H7; O114:H21; O115:H21; O126:H9; O128ac:H7; O128ac:H12; O128ac:H21; O148:H28; O149:H4; O159:H4; O159:H20; O166:H27; and O167:H5
Enteropathogenic (EPEC)	O26:NM; O26:H11; O55:NM; O55:H6; O86:NM; O86:H2; O86:H34; O111ab:NM; O111ab:H2; O111ab:H12; O111ab:H21; O114:H2; O119:H6; O125ac:H21; O127:NM; O127:H6; O127:H9; O127:H21; O128ab:H2; O142:H6; and O158:H23
Enteroinvasive (EIEC)	O28ac:NM; O29:NM; O112ac:NM; O115:NM; O124:NM; O124:H7; O124:H30; O135:NM; O136:NM; O143:NM; O144:NM; O152:NM; O164:NM; and O167:NM
Verotoxin-Producing (VTEC))	O1:NM; O2:H5; O2:H7; O4:NM; O4:H10; O5:NM; O5:H16; O6:H1; O18:NM; O18:H7; O25:NM; O26:NM; O26:H11; O26:H32; O38:H21; O39:H4; O45:H2; O50:H7; O55:H7; O55:H10; O82:H8; O84:H2; O91:NM; O91:H21; O103:H2; O111:NM; O111:H8; O111:H30; O111:H34; O113:H7; O113:H21; O114:H48; O115:H10; O117:H4; O118:H12; O118:H30; O121:NM; O121:H19; O125:NM; O125:H8; O126:NM; O126:H8; O128:NM; O128:H2; O128:H8; O128:H12; O128:H25; O145:NM; O125:H25; O146:H21; O153:H25; O157:NM; O157:H7; O163:H19; O165:NM; O165:19; and O165:H25

B. Verotoxin Producing Strains of *E. coli*

Although all of these disease-associated serotypes cause potentially life-threatening disease, *E. coli* O157:H7 and other verotoxin-producing strains have recently gained widespread public attention in the United States due to their recently recognized association with two serious extraintestinal diseases, hemolytic uremic syndrome ("HUS") and thrombotic thrombocytopenic purpura ("TTP"). Worldwide, *E. coli* O157:H7 and other verotoxin-producing *E. coli* (VTEC) are an increasingly important human health problem. First identified as a cause of human illness in early 1982 following two outbreaks of food-related hemorrhagic colitis in Oregon and Michigan (M.A. Karmali, "Infection by Verocytotoxin-Producing *Escherichia coli*," Clin. Microbiol. Rev., 2:15-38 [1989]; and L. W. Riley, *et al.* "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," New Eng. J. Med.,

308: 681-685 [1983]), the reported incidence of VTEC-associated disease has risen steadily, with outbreaks occurring in the U.S., Canada, and Europe.

With increased surveillance, *E. coli* O157:H7 has been recognized in other areas of the world including Mexico, China, Argentina, Belgium, and Thailand (N. V. Padhye and M. P. Doyle, "*Escherichia coli* O157:H7: Epidemiology, pathogenesis and methods for detection in food," J. Food. Prot., 55: 555-565 [1992]; and P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1991]).

The disease attracted national attention in the U.S. after a major outbreak in the Pacific Northwest that was associated with consumption of undercooked *E. coli* O157:H7-contaminated hamburgers. Over 700 hundred people fell ill (more than 170 were hospitalized) and four young children died (P. Recer, "Experts call for irradiation of meat to protect against food-borne bacteria," Associated Press, 7/12/94 [1994]). Several outbreaks since then have underscored the potential severity and multiple mechanisms for transmission of VTEC-associated diseases (M. Bielaszewská *et al.*, "Verotoxigenic (enterohaemorrhagic) *Escherichia coli* in infants and toddlers in Czechoslovakia," Infection 18: 352-356 [1990]; A. Caprioli *et al.*, "Hemolytic-uremic syndrome and Vero cytotoxin-producing *Escherichia coli* infection in Italy, "J. Infect. Dis., 166: 184-158 [1992]; A. Caprioli, *et al.*, "Community-wide Outbreak of Hemolytic-Uremic Syndrome Associated with Non-O157 Verocytotoxin-Producing *Escherichia coli*," J. Infect. Dis., 169: 208-211 [1994]; N. Cimolai, "Low frequency of high level Shiga-like toxin production in enteropathogenic *Escherichia coli* serogroups," Eur. J. Pediatr., 151: 147 [1992]; and R. Voelker., "Panel calls *E. coli* screening inadequate," *Escherichia coli* O157:H7--Panel sponsored by the American Gastroenterological Association Foundation in July 1994, Medical News & Perspectives, J. Amer. Med. Assoc., 272: 501 [1994]).

While O157:H7 is currently the predominant *E. coli* serotype associated with illness in North America, other serotypes (as shown in Table 1, and in particular O26:H11, O113:H21, O91:H21 and O111:NM) also produce verotoxins which appear to be important in the pathogenesis of gastrointestinal manifestations and the hemolytic uremic syndrome (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1990]; M. M. Levine, *et al.*, "Antibodies to Shiga holotoxin and to two synthetic peptides of the B subunit in sera of patients with *Shigella dysenteriae* 1

dysentery," J. Clin. Microbiol., 30: 1636-1641 [1992]; and C. R. Dorn. *et al.*, "Properties of Vero cytotoxin producing *Escherichia coli* of human and animal origin belonging to serotypes other than O157:H7," Epidemiol. Infect., 103: 83-95 [1989]). Since organisms with these serotypes have been shown to cause illness in humans they may assume greater public health importance over time (P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1990]).

Clinicians usually observe cases of hemolytic uremic syndrome ("HUS") clustered in a geographic region. However, small outbreaks are likely to be missed because many laboratories do not routinely screen stool specimens for *E. coli* O157:H7. Many cases related to non-commercial food preparation also probably go unrecognized. Nonetheless. *E. coli* O157:H7 is responsible for a large number of cases. as more than 20.000 cases of *E coli* O157:H7 infection are reported annually in the U.S.. with 400–500 deaths from HUS. However. these estimates were compiled when only 11 states mandated reporting of *E. coli* O157:H7. Twenty-nine states have recently made *E. coli* O157:H7 infection a reportable disease (R. Voelker. "Panel calls *E. coli* screening inadequate: *Escherichia coli* O157:H7; panel sponsored by the American Gastroenterological Association Foundation in July 1994. Medical News & Perspectives," J. Amer. Med. Assoc.. 272: 501 [1994]). Indeed. the Centers for Disease Control recently added *E. coli* O157:H7 to their list of reportable diseases ("Public Health Threats." Science 267:1427 [1995]).

C. Nature of Verotoxin-Induced Disease

Risk factors for HUS progression following infection with *E. coli* O157:H7 include age (very young or elderly), bloody diarrhea. leukocytosis. fever. large amounts of ingested pathogen. previous gastrectomy, and the use of antimicrobial agents (in particular, trimethoprim-sulfamethoxazole)(A. A. Harris *et al.*. "Results of a screening method used in a 12 month stool survey for *Escherichia coli* O157:H7." J. Infect. Dis.. 152: 775-777 [1985]; and M. A. Karmali. "Infection by Verocytotoxin-producing *Escherichia coli*." Clin. Microbiol. Rev., 2: 15-38 [1989]).

As indicated above. *E. coli* O157:H7 is associated with significant morbidity and mortality. The spectrum of illness associated with *E. coli* O157:H7 infection includes asymptomatic infection. mild uncomplicated diarrhea. hemorrhagic colitis. HUS. and TTP". Hemorrhagic colitis (or "ischemic colitis") is a distinct clinical syndrome characterized by

sudden onset of abdominal cramps—likened to the pain associated with labor or appendicitis—followed within 24 hours by watery diarrhea. One to two days later, the diarrhea turns grossly bloody in approximately 90% of patients and has been described as "all blood and no stool" (C. H. Pai *et al.*, "Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7," Ann. Intern. Med., 101: 738-742 [1984]; and R. S. Remis *et al.*, "Sporadic cases of hemorrhagic colitis associated with *Escherichia coli* O157:H7," Ann. Intern. Med., 101: 738-742 [1984]). Vomiting may occur, but there is little or no fever. The time from ingestion to first loose stool ranges from 3–9 days (with a mean of 4 days) L. W. Riley *et al.*, "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," New Eng. J. Med., 308: 681-685 [1983]; and D. Pudden *et al.*, "Hemorrhagic colitis in a nursing home," Ontario Can. Dis. Weekly Rpt., 11: 169-170 [1985]), and the duration of illness ranges generally from 2–9 days (with a mean of 4 days).

HUS is a life-threatening blood disorder that appears within 3–7 days following onset of diarrhea in 10–15% of patients. Those younger than 10 years and the elderly are at particular risk. Symptoms include renal glomerular damage, hemolytic anemia (rupturing of erythrocytes as they pass through damaged renal glomeruli), thrombocytopenia and acute kidney failure. Approximately 15% of patients with HUS die or suffer chronic renal failure. Indeed, HUS is a leading cause of renal failure in childhood (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 [1989]). Currently, blood transfusion and dialysis are the only therapies for HUS.

TTP shares similar histopathologic findings with HUS, but usually results in multiorgan microvascular thrombosis. Neurological signs and fever are more prominent in TTP, compared with HUS. Generally occurring in adults, TTP is characterized by microangiopathic hemolytic anemia, profound thrombocytopenia, fluctuating neurologic signs, fever and mild azotemia (H. C. Kwaan, "Clinicopathological features of thrombotic thrombocytopenic purpura," Semin. Hematol., 24: 71-81 [1987]; and S. J. Machin, "Clinical annotation: Thrombotic thrombocytopenic purpura," Br. J. Hematol., 56: 191-197 [1984]). Patients often die from microthrombi in the brain. In one review of 271 cases, a rapidly progressive course was noted, with 75% of patients dying within 90 days (E.L. Amorosi and J.E. Ultmann, "Thrombotic thrombocytopenic purpura: Report of 16 cases and review of the literature," Med., 45:139-159 (1966).

Other diseases associated with *E. coli* O157:H7 infection include hemorrhagic cystitis and balantitis (W. R. Grandsen *et al.*, "Hemorrhagic cystitis and balantitis associated with

verotoxin-producing *Escherichia coli* O157:H7." Lancet ii: 150 [1985]). convulsions. sepsis with other organisms and anemia (P. C. Rowe *et al.*, "Hemolytic anemia after childhood *Escherichia coli* O157:H7 infection: Are females at increased risk?" Epidemiol. Infect.. 106: 523-530 [1991]).

D. Mechanism of Pathogenesis

Verotoxins are strongly linked to *E. coli* O157:H7 pathogenesis. All clinical isolates of *E. coli* O157:H7 have been shown to produce one or both verotoxins (VT1 and VT2) (C. A. Bopp *et al.*, "Unusual Verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis." J. Clin. Microbiol.. 25: 1486-1489 [1987]). Both of these toxins are cytotoxic to Vero (African green monkey kidney) and HeLa cells. and cause paralysis and death in mice (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis." *Lancet* ii: 573 [1983]). These toxins are sometimes referred to in the literature as Shiga-like toxins I and II (SLT-I and SLT-II. respectively). due to their similarities with the toxins produced by *Shigella*. Indeed. much of our understanding of *E. coli* VTs is based on information accumulated on Shiga toxins. Shiga toxin. first described in 1903, has been recognized as one of the most potent bacterial toxins for eukaryotic cells (reviewed by M.A. Karmali. "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev.. 2: 15-38 [1989]). Hereinafter. the VT convention will be used; thus. VT1 and VT2 correspond to SLT-I and SLT-II. respectively.

While the pathogenic mechanism of *E. coli* O157:H7 infection is incompletely understood. it is believed that ingested organisms adhere to and colonize the intestinal mucosa. where toxins are released which cause endothelial cell damage and bloody diarrhea. It is also postulated that hemorrhagic colitis progresses to HUS when verotoxins enter the bloodstream. damaging the endothelial cells of the microvasculature and triggering a cascade of events resulting in thrombus deposition in small vessels. These microthrombi occlude the microcapillaries of the kidneys (particularly in the glomeruli) and other organs. resulting in their failure (J. J. Byrnes and J. L. Moake. "TTP and HUS syndrome: Evolving concepts of pathogenesis and therapy." Clin. Hematol., 15: 413-442 [1986]; and T. G. Cleary. "Cytotoxin-producing *Escherichia coli* and the hemolytic uremic syndrome." Pediatr. Clin. North Am.. 35: 485-501 [1988]). Verotoxins entering the bloodstream may also result in direct kidney cytotoxicity.

VT1 is immunologically and structurally indistinguishable from Shiga toxin produced by *Shigella dysenteriae* (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis," *Lancet* ii: 573 [1983]). VT1 and VT2 holotoxins each consist of one A and five B subunits (A. Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies," Infect. Immun., 57: 3888-3893 [1989]; and A. Donohue-Rolfe *et al.*, "Simplified high yield purification of *Shigella* toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies," J. Exp. Med., 160: 1767-1781 [1984]). The toxic A subunit is enzymatically active, while the B subunit binds the holotoxin to the receptor on the target eukaryotic cell.

Crystal structure analysis of Shiga holotoxin and VT1 B subunit pentamers have shown that the holotoxin assembles with the C-terminal end of the A subunit associating with, and inserting within, a pentamer of B chains (P. E. Stein *et al.*, "Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*," Nature 355: 748-750 [1992]; and M.E. Fraser *et al.*, "Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution," Struct. Biol., 1:59-64 [1994]). This conformation is consistent with the observation that a C-terminally truncated A1 subunit of VT1 is toxic (in a ribosomal inhibition assay), but cannot associate with B subunit pentamers (P. R. Austin *et al.* "Evidence that the A_2 fragment of Shiga-like toxin type I is required for holotoxin integrity," Infect. Immun., 62: 1768 [1994]).

The Verotoxin A Subunit. Examination of the crystal structure of Shiga holotoxin indicates that the N-terminus of its A subunit is both surface-exposed and functionally important. Removal of amino acid interval 3–18 of the A subunit completely abolished toxicity (L. P. Perera *et al.*, "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II," Infect. Immun., 59: 829-835 [1991]) while removal of interval 25–44 retained toxicity but abolished its association with B subunit pentamers (J. E. Haddad *et al.*, "Minimum domain of the Shiga toxin A subunit required for enzymatic activity," J. Bacteriol., 175: 4970-4978 [1993]). Deletion of the first 13 residues of the homologous ricin A subunit also abolished toxicity, while deletion of the first 9 residues did not (M. J. May, *et al.*, "Ribosome inactivation by ricin A chain: A sensitive method to assess the activity of wild-type and mutant polypeptides. " EMBO J., 8: 301-308 [1989]).

The Verotoxin B Subunit. Studies of Shiga toxin B subunit suggest that neutralizing epitopes may also be present at both the N- and C-terminal regions of VT1 and VT2 B

subunits. Polyclonal antibodies raised against peptides from these regions (residues 5–18. 13–26. 7–26. 54–67 and 57–67) show partial neutralization of Shiga toxin (I. Harari and R. Arnon. "Carboxy-terminal peptides from the B subunit of Shiga toxin induce a local and parenteral protective effect," Mol. Immunol., 27: 613-621 [1990]: and I. Harari *et al.*, "Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity," Infect. Immun., 56: 1618-1624 [1988]). Deletion of the last five amino acids of Shiga toxin B (M. P. Jackson *et al.*. "Functional Analysis of the Shiga toxin and Shiga-like toxin Type II variant binding subunits by using site-directed mutagenesis." J. Bacteriol.. 172: 653-658 [1990]). or four amino acids of VT2 B (L. P. Perera *et al.*. "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II." Infect. Immun.. 59: 829-835 [1991]), eliminate toxin activity, while deletion of the last two amino acids of VT2 B subunit reduced cytotoxicity. In contrast. the addition of an 18 or 21 amino acid extension to the native C-terminus of the VT2 B subunit was presumably conformationally correct. as these proteins assembled cytotoxic holotoxin.

Various approaches to express recombinant verotoxins have included individual or coordinate expression of A and B subunits from high-copy number plasmids and expression with fusion partners (J. E. Haddad *et al.*, "Minimum domain of the Shiga toxin A subunit required for enzymatic activity." J. Bacteriol., 175: 4970-4978 ; J. E. Haddad. and M. P. Jackson. "Identification of the Shiga toxin A-subunit residues required for holotoxin assembly." J. Bacteriol.. 175: 7652-7657 [1993]: M. P. Jackson *et al.*. "Mutational analysis of the Shiga toxin and Shiga-like toxin II enzymatic subunits." J. Bacteriol.. 172: 3346-3350 [1990]: C. J. Hovde *et al.*. "Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I." Proc. Natl. Acad. Sci., 85: 2568-2572 [1988]: R. L. Deresiewicz *et al.*. "The role of tyrosine-114 in the enzymatic activity of the Shiga-like toxin I A-chain." Mol. Gen. Genet.. 241: 467-473 [1993]; T. M. Zollman *et al.*. "Purification of Recombinant Shiga-like Toxin Type I A_1 Fragment from *Escherichia coli*." Protein Express.Purific.. 5: 291-295 [1994]; K. Ramotar. *et al.*. "Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron." Biochem J.. 272: 805-811 [1990]: S. B. Calderwood *et al.*. "A system for production and rapid purification of large amounts of the Shiga toxin/Shiga-like toxin I B subunit," Infect. Immun.. 58: 2977-2982 [1990]; D. W. K. Acheson. *et al.*. "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]; M. P. Jackson *et al.*. "Nucleotide sequence analysis and

comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," FEMS Microbiol. Lett., 44: 109-114 [1987]; J. W. Newland *et al.*, "Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II." Infect. Immun. 55: 2675-2680 [1987]; and F. Gunzer and H. Karch, "Expression of A and B subunits of Shiga-like toxin II as fusions with glutathione *S*-transferase and their potential for use in seroepidemiology.". J. Clin. Microbiol., 31: 2604-2610 [1993]; and D.W. Acheson *et al.*, "Expression and purification of Shiga-like toxin II B subunits." Inf. Immun., 63:301-308 [1995]). In one case, bench top fermentation techniques yielded 22 mg/liter of soluble recombinant protein (D. W. K. Acheson, *et al.*, "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]). However, there have been no systematic approaches to identifying the appropriate spectrum of VT antigens, preserving immunogen and immunoabsorbant antigenicity and scaling-up.

The receptor for VT1 and VT2 is a globotriaosyl ceramide containing a galactose α-(1-4)- galactose-β-(1-4) glucose ceramide (Gb3) (C. A. Lingwood *et al.*, "Glycolipid binding of natural and recombinant *Escherichia coli* produced verotoxin *in vitro*," J. Biol. Chem., 262: 1779-1785 [1987]; and T. Wadell *et al.*, "Globotriaosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2." Biochem. Biophys. Res. Commun., 152: 674-679 [1987]). Gb3 is abundant in the cortex of the human kidney and is present in primary human endothelial cell cultures. Hence, the identification of Gb3 as the functional receptor for VT1 and VT2 is consistent with their role in HUS pathogenesis, in which endothelial cells of the renal vasculature are the principal site of damage. Therefore, toxin-mediated pathogenesis may follow a sequence of B subunit binding to Gb3 receptors on kidney cells, toxin internalization, enzymatic reduction of the A subunit to an A1 fragment, binding of the A1 subunit to the 60S ribosomal subunit, inhibition of protein synthesis and cell death (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]).

The role of verotoxins in the pathogenesis of *E. coli* O157:H7 infections has been further studied in animal models. Infection or toxin challenge of laboratory animals do not produce all the pathologies and symptoms of hemorrhagic colitis, HUS, and TTP which occur in humans. Glomerular damage is noticeably absent. Nonetheless, experiments using animal models implicate verotoxins as the direct cause of hemorrhagic colitis, microvascular damage leading to the failure of kidneys and other organs and CNS neuropathies.

For example, Barrett, *et al.* delivered VT2 into the peritoneal cavity of rabbits using mini-osmotic pumps (J. J. Barrett *et al.*, "Continuous peritoneal infusion of shiga-like toxin II (SLTII) as a model for SLT II-induced diseases," J. Infect. Dis., 159: 774-777 [1989]). In three days, most animals receiving the toxin developed diarrhea, with intestinal lesions resembling those seen in humans with hemorrhagic colitis. Although there was some evidence of renal dysfunction, none of the rabbits developed HUS. Beery, *et al.* showed that VT2, when administered intraperitoneally or intravenously to adult mice, produces lesions of the kidneys and colon (J. T. Beery *et al.*, "Cytotoxic activity of *Escherichia coli* O157:H7 culture filtrate on the mouse colon and kidney," Curr. Microbiol., 11: 335-342 [1984]). Histologic lesions in the kidney included accumulation of numerous exfoliated collecting tubules and marked intracellular vacuolation of proximal convoluted tubular cells.

Sjögren *et. al.* studied the pathogenesis of an entero-adherent strain of *E. coli* (RDEC-1) lysogenized with a VT1-containing bacteriophage (VT1-producing RDEC-1) (R. Sjögren *et al.*, "Role of Shiga-like toxin I in bacterial enteritis: comparison between isogenic *Escherichia coli* strains induced in rabbits," Gastroenterol., 106: 306-317 [1994]). In this study, rabbits were challenged with RDEC-1 or VT1-producing RDEC-1 and studied for onset of disease. The VT1-producing variant induced a severe, non-invasive, entero-adherent infection in rabbits which was characterized by serious histological lesions with vascular changes, edema and severe epithelial inflammation. Importantly, vascular changes consistent with endothelial damage were seen in infected animals that was similar to intestinal microvascular changes in humans with *E. coli* O157:H7 infection. Based on these observations, they concluded that VT1 is an important virulence factor in enterohemorrhagic *E. coli* O157:H7 infection.

Fuji *et. al.* described a model in which mice were treated for three days with streptomycin followed by a simultaneous challenge of *E. coli* O157:H7 orally, and mitomycin intraperitoneally (J. Fuji *et al.*, "Direct evidence of neuron impairment by oral infection with Verotoxin-producing *Escherichia coli* O157:H7 in mitomycin-treated mice," Infect. Immun., 62: 3447-34453 [1994]). All of the animals died within four days. Immunoelectron-microscopy strongly suggested that death was due to the toxic effects of VT2v (a structural variant of VT2), on both the endothelial cells and neurons in the central nervous system which resulted in fatal acute encephalopathy.

Wadolkowski *et al.* studied colonization of *E. coli* O157:H7 in mice. Mice were treated with streptomycin and fed 10^{10} *E. coli* O157:H7 (E. A. Wadolkowski *et al.*, "Mouse

model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun.. 58: 2438-2445 [1990]; and E. A. Wadolkowski *et al.*. "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]). All of the mice died due to severe. disseminated. acute necrosis of proximal convoluted tubules. In mouse models. glomerular damage was not observed. but toxic acute renal tubular necrosis was observed which is characteristic of some HUS patients. The failure of mice to show glomerular damage is thought to be due to the absence of a functional globotriaosyl ceramide receptor specific for verotoxins in the glomeruli of the kidneys. Administration of VT2 subunit-specific monoclonal antibodies prior to infection prevented all pathology and death.

E. Current Therapeutic Approaches

E. coli O157:H7 disease is not adequately controlled by current therapy. Patient treatment is tailored to manage fluid and electrolyte disturbances. anemia. renal failure and hypertension. Although *E. coli* O157:H7 is susceptible to common antibiotics. the role of antibiotics in the treatment of infection has questionable merit. In both retrospective and prospective studies. prophylaxis or treatment with antibiotics such as trimethoprim-sulfamethoxazole. there was either no benefit or an increased risk of developing HUS (T. N. Bokete *et al.*. "Shiga-like toxin producing *Escherichia coli* in Seattle children: a prospective study." Gastroenterol.. 105: 1724-1731 [1993]; A. T. Pavia *et al.*. "Hemolytic uremic-syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations." J. Pedatr.. 116: 544-551 [1990]; F. Proulx *et al.*. "Randomized. controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis." J. Pediatr. 121: 299-303 [1992]; and A. L. Carter *et al.*. "A severe outbreak of *Escherichia coli* O157:H7-associated hemorrhagic colitis in a nursing home." New Eng. J. Med.. 317: 1496-1500 [1987]).

The mechanisms by which antibiotics increase the risk of infection or related complications might involve enhancement of toxin production. release of toxins from killed organisms. or alteration of normal competing intestinal flora allowing for pathogen overgrowth (M. A. Karmali. "Infection by Verocytotoxin-producing *Escherichia coli*." Clin. Microbiol. Rev.. 2: 15-38 [1989]). A further concern in the use of antibiotics is the potential acquisition of antimicrobial resistance by *E. coli* O157:H7 (C. R. Dorn. "Review of foodborne outbreak of *Escherichia coli* O157:H7 infection in the western United States." JAVMA 203: 1583-1587 [1993]).

In addition, by the time symptoms are serious enough to attract medical attention, it is likely that verotoxins are already entering the systemic circulation or will do so shortly thereafter. Although antimicrobials may help to prevent pathology resulting from the action of toxin on the bowel lumen. However, by the time symptoms of HUS have developed, the patient has ceased shedding organisms. Thus, antimicrobial treatment during HUS disease is of less value, and often contraindicated, due to the increased risk of complications associated with administration of antimicrobials to patients susceptible to development of HUS. Importantly, there is currently no antitoxin commercially available for use in treating affected patients. What is needed is a means to block the progression of disease, without the complications associated with antimicrobial treatment.

DESCRIPTION OF THE DRAWINGS

Figure 1 is an SDS-PAGE of rVT1 and rVT2.

Figure 2 shows HPLC results for rVT1 and rVT2.

Figure 3 shows rVT1 and rVT2 toxicity in Vero cell culture.

Figure 4 shows EIA reactivity of rVT1 and rVT2 antibodies to rVT1.

Figure 5 shows EIA reactivity of rVT1 and rVT2 Antibodies to rVT2.

Figure 6 shows Western Blot reactivity of rVT1 and rVT2 antibodies to rVT's:

Panel 6A contains preimmune IgY;
Panel 6B contains rVT1 IgY; and
Panel 6C contains rVT2 IgY.

Figure 7 shows neutralization of rVT1 cytotoxicity in Vero cells.

Figure 8 shows neutralization of rVT2 cytotoxicity in Vero cells.

Figure 9 shows renal sections from *E. coli* O157:H7-infected mice treated with IgY

Panel 9A shows a representative kidney section from a mouse treated with preimmune IgY;
Panel 9B shows a representative kidney sections from a mouse treated with rVT1; and
Panel 9C shows a representative kidney section from a mouse treated with rVT2 IgY.

Figure 10 shows the fusion constructs of VT components and affinity tags.

DEFINITIONS

To facilitate understanding of the invention. a number of terms are defined below.

As used herein. the term "neutralizing" is used in reference to antitoxins. particularly antitoxins comprising antibodies. which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein. the term "overproducing" is used in reference to the production of toxin polypeptides in a host cell. and indicates that the host cell is producing more of the toxin by virtue of the introduction of nucleic acid sequences encoding the toxin polypeptide than would be expressed by the host cell absent the introduction of these nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce the toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

As used herein. the term "fusion protein" refers to a chimeric protein containing the protein of interest (*i.e.*, an *E. coli* verotoxin and/or fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the *E. coli* protein as expressed in a host cell. may provide an "affinity tag" to allow purification of the recombinant fusion protein from the host cell or culture supernatant. or both. If desired. the fusion protein may be removed from the protein of interest (*i.e.*, toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein. the term "affinity tag" refers to such structures as a "poly-histidine tract" or "poly-histidine tag." or any other structure or compound which facilitates the purification of a recombinant fusion protein from a host cell. host cell culture supernatant. or both. As used herein. the term "flag tag" refers to short polypeptide marker sequence useful for recombinant protein identification and purification.

As used herein. the terms "poly-histidine tract" and "poly-histidine tag." when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

As used herein. the term "chimeric protein" refers to two or more coding sequences obtained from different genes. that have been cloned together and that. after translation. act as

a single polypeptide sequence. Chimeric proteins are also referred to as "hybrid proteins." As used herein, the term "chimeric protein" refers to coding sequences that are obtained from different species of organisms, as well as coding sequences that are obtained from the same species of organisms.

As used herein, the term "protein of interest" refers to the protein whose expression is desired within the fusion protein. In a fusion protein, the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

As used herein, the term "maltose binding protein" and "MBP" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of substantially all immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins; the percent of recombinant toxin polypeptides is thereby increased in the sample.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell. is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence. the soluble protein is exported to the periplasmic space in bacterial hosts and is secreted into the culture medium of eukaryotic cells capable of secretion or by bacterial hosts possessing the appropriate genes. In contrast. an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion bodies) in the host cell. High level expression (*i.e.*. greater than 1 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

A distinction is drawn between a soluble protein (*i.e.*. a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (*i.e.*. rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride. urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However. dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out): therefore. SDS-solubilized inclusion body protein is soluble but not refolded.

As used herein. the term "reporter reagent" or "reporter molecule" is used in reference to compounds which are capable of detecting the presence of antibody bound to antigen. For example. a reporter reagent may be a colorimetric substance which is attached to an enzymatic substrate. Upon binding of antibody and antigen. the enzyme acts on its substrate and causes the production of a color. Other reporter reagents include. but are not limited to fluorogenic and radioactive compounds or molecules.

As used herein the term "signal" is used in reference to the production of a sign that a reaction has occurred. for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorogenic reactions. and enzymatic reactions will be

used with the present invention. The signal may be assessed quantitatively as well as qualitatively.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of *E. coli* toxin in a subject.

As used herein, the term "acute intoxication" is used in reference to cases of *E. coli* infection in which the patient is currently suffering from the effects of toxin (*e.g.*, *E. coli* verotoxins or enterotoxins). Signs and symptoms of intoxication with the toxin may be immediately apparent. Or, the determination of intoxication may require additional testing, such as detection of toxin present in the patient's fecal material.

As used herein, the term "at risk" is used in references to individuals who have been exposed to *E. coli* and may suffer the symptoms associated with infection or disease with these organisms, especially due to the effects of verotoxins.

SUMMARY OF THE INVENTION

The present invention relates to antitoxin therapy for humans and other animals. Antitoxins which neutralize the pathologic effects of *E. coli* toxins are generated by immunization of avian hosts with recombinant toxin fragments. In one embodiment, the present invention contemplates a method of treatment administering at least one antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable to an intoxicated subject. It is contemplated that the intoxicated subject will be either an adult or a child.

In a preferred embodiment, the *E. coli* verotoxin is recombinant. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence. In one embodiment of the *E. coli* fusion protein, the fusion protein comprises a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

Various routes of administration, are contemplated for providing the *E. coli* antitoxin(s) to an affected individual, including but not limited to, parenteral as well as oral routes of administration. In a particularly preferred embodiment, the route of administration is parenteral.

The present invention also includes the embodiment of a method of prophylactic treatment in which an antitoxin directed against at least one *E. coli* verotoxin in an aqueous

solution in therapeutic amount that is parenterally administrable. and is administered to at least one subject at risk of diarrheal disease. It one embodiment. the antitoxin is parenterally administered.

In one embodiment. the subject is at risk of developing extra-intestinal complications of *E. coli* infections. including but not limited to. hemolytic uremic syndrome. thrombotic thrombocytopenic purpura. etc.

The present invention also includes the embodiment of a composition which comprises neutralizing antitoxin directed against at least one *E. coli* verotoxin in an aqueous solution in therapeutic amounts. In one particularly preferred embodiment. the *E. coli* verotoxin is a recombinant toxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT1 sequence. In another embodiment. the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT2 sequence. In yet another embodiment. the composition of the antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment. the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1. In an alternative embodiment. the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2. Indeed. the invention contemplates an antitoxin that is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment. the antitoxin is an avian antitoxin.

The present invention also comprises a method of treatment of enteric bacterial infections comprising administering an avian antitoxin directed against at least one verotoxin produced by *E. coli* in an aqueous solution in therapeutic amount. to at least one infected subject. In one preferred embodiment. the avian antitoxin is administered parenterally.

In another embodiment. the *E. coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7, O1:NM; O2:H5: O2:H7; O4:NM: O4:H10; O5:NM; O5:H16: O6:H1; O18:NM; O18:H7; O25:NM; O26:NM: O26:H11: O26:H32: O38:H21: O39:H4: O45:H2; O50:H7: O55:H7; O55:H10; O82:H8: O84:H2: O91:NM: O91:H21; O103:H2: O111:NM: O111:H8; O111:H30; O111:H34: O113:H7; O113:H21; O114:H48; O115:H10: O117:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM; O125:H8: O126:NM: O126:H8: O128:NM; O128:H2: O128:H8: O128:H12: O128:H25: O145:NM; O125:H25: O146:H21; O153:H25: O157:NM: O163:H19: O165:NM: O165:19: and O165:H25. In one embodiment. the antitoxin comprises antitoxin directed against at least one

Escherichia coli verotoxin. In another embodiment. the antitoxin is cross-reacts with at least one *Escherichia coli* verotoxin. In yet another embodiment. the antitoxin is reactive against toxins produced by members of the genus *Shigella*. including *S. dysenteriae*.

The present invention also contemplates uses for the toxin fragments in vaccines and diagnostic assays. The fragments may be used separately as purified. soluble antigens or. alternatively. in mixtures or "cocktails." The present invention thus comprises a method for detecting *Escherichia coli* verotoxin in a sample in which a sample.an antitoxin raised against *Escherichia coli* verotoxin. and a reporter reagent capable of binding the antitoxin are provided. The antitoxin is added to the sample, so that the antitoxin binds to the *E. coli* verotoxin in the sample. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment. the method further comprises the steps of washing unbound antitoxin from the sample. adding at least one reporter reagent to the sample. so that said reporter reagent binds to any antitoxin that is bound. washing the unbound reporter reagent from the sample and detecting the reporter reagent bound to the antitoxin bound to the *Escherichia coli* verotoxin. so that the verotoxin is detected. In one embodiment. the detecting is accomplished through any means. such as enzyme immunoassay. radioimmunoassay. fluorescence immunoassay, flocculation. particle agglutination. and *in situ* chromogenic assay. In one preferred embodiment, the sample is a biological sample. In an alternative preferred embodiment. the sample is an environmental sample.

DESCRIPTION OF THE INVENTION

The present invention contemplates treating humans and other animals intoxicated with at least one bacterial toxin. It is contemplated that administration of antitoxin will be used to treat patients effected by or at risk of symptoms due to the action of bacterial toxins. It is also contemplated that the antitoxin will be used in a diagnostic assay to detect the presence of toxins in samples. The organisms. toxins and individual steps of the present invention are described separately below.

I. Antibodies Directed Against *E. coli* and Associated Toxins

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against various *E. coli* serotypes, their toxins. enzymes or other metabolic by-products. cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization

of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment. toxins from all *E. coli* serotypes are contemplated as immunogens. Examples of these toxins include the verotoxins VT1 and VT2.

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin may be used as an effective therapeutic against one or more toxin(s) produced by other *E. coli* serotypes. or other toxin producing organisms (*e.g.*. *Shigella*. *Bacillus cereus*. *Staphylococcus aureus*. *Streptococcus mutans*. *Acinetobacter calcoaceticus*. *Pseudomonas aeruginosa*. other *Pseudomonas* species. *Vibrio* species. *Clostridium* species. etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

II. Obtaining Antibodies In Non-Mammals

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However. it is also contemplated that antibodies may be obtained from non-mammals without immunization. In the case where no immunization is contemplated. the present invention may use non-mammals with preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

In a preferred embodiment. the method of the present invention contemplates immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment. toxins from all *E. coli* serotypes are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (*i.e.*. recombinant toxin proteins). In a preferred embodiment. the immunogen comprises recombinant VT1 and/or VT2.

When immunization is used. the preferred non-mammal is from the class *Aves*. All birds are contemplated (*e.g.*. duck. ostrich. emu. turkey. etc.). A preferred bird is a chicken. Importantly. chicken antibody does not fix mammalian complement (*See* H.N. Benson *et al.*.

J. Immunol. 87:616 [1961]). Thus, chicken antibody will normally not cause a complement-dependent reaction (A.A. Benedict and K. Yamaga. *"Immunoglobulins and Antibody Production in Avian Species," in Comparative Immunology* (J.J. Marchaloni. ed.), pp. 335-375, Blackwell, Oxford [1966]). Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins presently known.

When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum (*See* R. Patterson *et al.*, J. Immunol. 89:272 (1962); and S.B. Carroll and B.D. Stollar. J. Biol. Chem. 258:24 [1983]). In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is more pure and more homogeneous: there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. In one embodiment, glutaraldehyde treatment of the toxin is contemplated. In an alternative embodiment, formaldehyde treatment of the toxin is contemplated.

It is not intended that the present invention be limited to a particular mode of immunization: the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. As used herein, the term "adjuvant" is defined as a substance known to increase the immune response to other antigens when administered with other antigens. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of

adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. The invention also contemplates the use of fowl adjuvant commercially available from RIBI, as well as Quil A adjuvant commercially available from Accurate Chemical and Scientific Corporation, and Gerbu adjuvant also commercially available (GmDP; C.C. Biotech Corp.).

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 35.

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be buffer-extracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (*e.g.*, immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000 (Polson *et al.*, Immunol. Comm. 9:495 [1980]). The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly more pure, in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed,

PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

IV. Treatment

The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by parenteral administration of antitoxin.

A. Dosage Of Antitoxin

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (*e.g*, horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies; and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment, the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non (mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

As is true in cases of botulism, the degree of an individual's exposure to *E. coli* toxin and the prognosis are often difficult to assess, and depend upon a number of factors (*e.g.*, the quantity of contaminated food ingested, the toxigenicity and serotype of *E. coli* strain ingested, etc.). Thus, the clinical presentation of a patient is usually a more important consideration than a quantitative diagnostic test, for determination of dosage in antitoxin

administration. Indeed, for many toxin-associated diseases (*e.g.*, botulism, tetanus, diphtheria, etc.), there is no rapid, quantitative test to detect the presence of the toxin or organism. Rather, these toxin-associated diseases are medical emergencies which mandate immediate treatment. Confirmation of the etiologic agent must not delay the institution of therapy, as the condition of an affected patient may rapidly deteriorate. In addition to the initial treatment with antitoxin, subsequent doses may be indicated, as the patient's disease progresses. The dosage and timing of these subsequent doses is dependent upon the signs and symptoms of disease in each individual patient.

It is contemplated that the administration of antitoxin to an affected individual would involve an initial injection of an approximately 10 ml dose of immune globulin (with less than approximately 1 gram of total protein). In one preferred embodiment, it is contemplated that at least 50% of the initial injection comprises immune globulin. It is also contemplated that more purified immune globulin be used for treatment, wherein approximately 90% of the initial injection comprises immune globulin. When more purified immune globulin is used, it is contemplated that the total protein will be less than approximately 100 milligrams. It is also contemplated that additional doses be given, depending upon the signs and symptoms associated with *E. coli* verotoxin disease progression.

B. Delivery Of Antitoxin

Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is parenteral or oral.

In one embodiment, antitoxin is parenterally administered to a subject in an aqueous solution. It is not intended that the parenteral administration be limited to a particular route. Indeed, it is contemplated that all routes of parenteral administration will be used. In one embodiment, parenteral administration is accomplished via intramuscular injection. In an alternative embodiment, parenteral administration is accomplished via intravenous injection.

In another embodiment, antitoxin is delivered in a solid form (*e.g.*, tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (*e.g.*, carbonate buffer, pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an

aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant or a dietary supplement formula (*e.g.*, Similac®, Ensure®, and Enfamil®). Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

Methods of applying enteric coatings to pharmaceutical compounds are well known to the art (companies specializing in the coating of pharmaceutical compounds are available: for example, The Coating Place [Verona, WI] and AAI [Wilmington, NC]). Enteric coatings which are resistant to gastric fluid and whose release (*i.e.*, dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available (for example, the polymethacrylates Eudragit® L and Eudragit® S [Röhm Tech Inc., Malden, MA]). Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause them to dissolve thereby releasing the antitoxin.

The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin. In another embodiment of treatment of acute intoxication, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (*i.e.*, a suspension) to recipients unable to tolerate administration of tablets (*e.g.*, children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic

dosage. In yet another preferred embodiment of prophylactic treatment. a therapeutic dosage of the antitoxin in a delivery solution. is parenterally administered.

V. Multivalent Vaccines Against *E. coli* Strains

The invention contemplates the generation of multivalent vaccines for the protection of an organism (particularly humans) against several *E. coli* strains. Of particular interest is a vaccine which stimulates the production of a humoral immune response to *E. coli* O157:H7, O26:H11, O113:H21. O91:H21, and O111:NM, in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the *E. coli* serotypes listed above. When native toxin proteins are used as immunogens they are generally modified to reduce the toxicity. It is contemplated that glutaraldehyde-modified toxin proteins will be used. In an alternative embodiment. is formaldehyde-modified toxin proteins will be used.

The invention contemplates that recombinant *E. coli* verotoxin proteins be used in conjunction with either native toxins or toxoids from other organisms as antigens in a multivalent vaccine preparation. It is also contemplated that recombinant *E. coli* toxin proteins be used in the multivalent vaccine preparation.

VI. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture (*e.g.*, microbiological cultures). On the other hand. it is meant to include both biological and environmental samples.

Biological samples may be animal. including human. fluid. solid (*e.g.*, stool) or tissue. as well as liquid and solid food and feed products and ingredients such as dairy items. vegetables. meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of common domestic animals, including but not limited to bovines (*e.g.* cattle). ovines (*e.g.*, sheep), caprines (*e.g.*, goats). porcines (*e.g.*, swine). equines (*e.g.*, horses). canines (*e.g.*, dogs), lagamorphs (*e.g.*, rabbits), and felines (*e.g.*, cats), etc. It is also intended that samples may be obtained from feral or wild animals. including, but not limited to. such animals as ungulates (*e.g.*, deer). bear. fish. lagamorphs. rodents. etc.

Environmental samples include environmental material such as surface matter. soil. water and industrial samples. as well as samples obtained from food and dairy processing

instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin VT1 and toxin VT2 proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (*e.g.*, a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (*e.g.*, microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (*e.g.*, soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following

the exposure of the liquid to the immobilized antibody. unbound toxin is substantially removed by washing. The liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme. fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (*i.e.*, in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (*e.g.*, the addition of enzyme substrate in enzyme systems: observation using fluorescent light for fluorescent dye systems: and quantitation of radioactivity for radioactive systems).

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows. the following abbreviations apply: °C (degrees Centigrade): rpm (revolutions per minute); BSA (bovine serum albumin): ELISA (enzyme-linked immunosorbent assay): IgG (immunoglobulin G); IgY (immunoglobulin Y): IP (intraperitoneal): SC (subcutaneous): H_2O (water): HCl (hydrochloric acid): LD_{100} (lethal dose for 100% of experimental animals): aa (amino acid): HPLC (high performance liquid chromatography): Kda (kilodaltons): gm (grams); μg (micrograms): mg (milligrams); ng (nanograms): μl (microliters): ml (milliliters): mm (millimeters); nm (nanometers): μm (micrometer): M (molar): mM (millimolar); MW (molecular weight); sec (seconds): min(s) (minute/minutes): hr(s) (hour/hours): $MgCl_2$ (magnesium chloride): NaCl (sodium chloride): Na_2CO_3 (sodium carbonate): OD_{280} (optical density at 280 nm): OD_{600} (optical density at 600 nm): PAGE (polyacrylamide gel electrophoresis): PBS [phosphate buffered saline (150 mM NaCl. 10 mM sodium phosphate buffer. pH 7.2)]; PEG (polyethylene glycol): SDS (sodium dodecyl sulfate): Tris (tris(hydroxymethyl)aminomethane): w/v (weight to volume): v/v (volume to volume): Amicon (Amicon. Inc.. Beverly, MA): Amresco (Amresco. Inc.. Solon. OH); ATCC (American Type Culture Collection. Rockville. MD); BBL (Baltimore Biologics Laboratory. (a division of Becton Dickinson). Cockeysville. MD): Becton Dickinson (Becton Dickinson Labware. Lincoln Park. NJ); BioRad (BioRad. Richmond. CA): Biotech (C-C Biotech Corp.. Poway. CA): Charles River (Charles River Laboratories. Wilmington. MA): Falcon (*e.g.* Baxter Healthcare Corp.. McGaw Park. IL and Becton Dickinson); Fisher Biotech (Fisher Biotech. Springfield. NJ); GIBCO (Grand Island Biologic Company/BRL. Grand Island, NY): Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL);

Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs. Inc.. Beverly. MA); Novagen (Novagen, Inc., Madison. WI); Pharmacia (Pharmacia. Inc., Piscataway, NJ); Qiagen (Qiagen, Chatsworth, CA); Showdex (Showa Denko America. Inc.. New York. NY); Sigma (Sigma Chemical Co., St. Louis. MO); RIBI (RIBI Immunochemical Research Inc.. Hamilton. MT); Accurate Chemical and Scientific Corp. (Accurate Chemical and Scientific Corp.. Hicksville, NY); Kodak (Eastman-Kodak. Rochester. NY); and Stratagene (Stratagene. La Jolla. CA).

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. The specification gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

The first set of Examples (Examples 1-5) was designed to develop an antidote to *E. coli* O157:H7 verotoxins and evaluate its effectiveness *in vitro* and *in vivo*. In the first experiments. high titer verotoxin antibodies were generated in laying hens hyperimmunized with chemically detoxified and/or native verotoxins. These Laying hens were immunized with either recombinant *E. coli* O157:H7 VT1 or VT2 (rVT1 and rVT2) treated with glutaraldehyde and mixed with adjuvant.

Next. toxin-reactive polyclonal antibodies were isolated by bulk fractionation from egg yolks pooled from hyperimmunized hens. Large quantities of polyclonal antibodies (IgY) were harvested from resulting eggs using a two-step polyethylene glycol fractionation procedure.

Third. the immunoreactivity and yields of VT IgY were analyzed by analytical immunochemical methods (*e.g.*, enzyme immunoassay (EIA) and Western blotting). EIA and Western blot analysis showed that the resulting egg preparations contained high titer IgY that reacted with both the immunizing and the heterologous toxins (*i.e.*, rVT1 IgY reacted against both rVT1 and rVT2. and vice versa).

Fourth. VT neutralization potency was analyzed *in vitro* using a Vero cytotoxicity assay. Vero cytotoxicity of rVT1 and rVT2 could be completely inhibited by VT IgY. These antibodies also demonstrated substantial verotoxin cross-neutralization.

Fifth. the efficacy of passively administered avian verotoxin antibodies in preventing the lethal effects of verotoxin poisoning was assessed in a mouse disease model. Toxin neutralizing antibodies were administered by parenteral dosing regimens to assess the most

effective strategy for therapeutic intervention. Efficacy of verotoxin antibodies was demonstrated using multiple murine disease models. In these experiments, antibodies prevented both the morbidity and lethality of homologous and heterologous toxins using a toxin/antitoxin premix format; mice infected orally with a lethal dose of viable *E. coli* O157:H7 were protected from both morbidity and lethality when treated parenterally four hours post-infection with either rVT1 or rVT2 antibodies; and mice given a lethal dose of *E. coli* O91:H21 (a particularly virulent strain which only produces VT2c, a VT2 structural variant) and treated parenterally *up to 10 hours later* with rVT1 IgY administered parenterally were protected from both morbidity and lethality.

EXAMPLE 1
TOXIN ANALYSIS AND IMMUNIZATION

Purified recombinant *E. coli* O157:H7 verotoxins, rVT1 and rVT2, were obtained from Denka Sieken Co., Ltd. (Tokyo, Japan). Toxin genes were isolated, inserted into expression plasmids, and expressed in *E. coli*. Recombinant proteins were then purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE Sephacryl and hydroxyapatite, and gel filtration chromatography by the supplier. Upon receipt, toxins were analyzed to verify identity, purity and toxicity, as described below.

A. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Samples of each toxin (2 μg) were heat-denatured in a buffer containing SDS and β-mercaptoethanol followed by electrophoresis on 10–20% gradient gels (Bio-Rad, Richmond, CA). Resolved polypeptide bands were visualized using the silver stain procedure of C.R. Merril, *et al.*, "Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins," Science 211: 1437-1438 (1981).

VT1 and VT2 are each composed of subunit A and multiple copies of subunit B. Subunit A is often nicked into fragments A1 and A2 which are linked by a disulfide bridge. As shown in Figure 1, when separated by SDS-PAGE in the presence of β-mercaptoethanol, rVT1 resolved into 3 bands that corresponded to subunit A (~31 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~4 Kda). Similarly, rVT2 resolved into subunit A (~33 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~8 Kda) (Figure 1). In this Figure, rVT1 is in Lane 1, and rVT2 is in Lane 2; the positions of

molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

These results are consistent with previous reports of VT1 and VT2 purified from naturally occurring toxigenic strains (V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." Biochem. Biophys. Res. Commun., 139: 424-430 [1986]; and F. B. Kittel *et al.*. "Characterization and inactivation of verotoxin 1 produced by *Escherichia coli* O157:H7." J. Agr. Food Chem., 39: 141-145 [1991]).

B. High Performance Liquid Chromatography (HPLC).

Chromatography was performed at room temperature (RT) under isocratic conditions using a Waters 510 HPLC pump. Eluted protein was measured using a Waters 490E programmable multi-wavelength detector (Millipore Corp., Milford, MA). The VT's were separated on an 8 x 300 mm (ID) Shodex KW803 column, using 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (phosphate buffered saline [PBS]) as the mobile phase at a flow rate of 1 ml/min.

The purity of non-denatured rVT's was assessed by HPLC. As shown in the chromatographs in Figure 2, each toxin eluted at approximately 10 min. as a single absorbance peak at 280 nm. By integration of the area under each peak, the rVT's were shown to be >99% pure.

C. Vero Cell Cytotoxicity Assay.

Cytotoxic activity of rVT1 and rVT2 was assessed using modified procedures of Padhye, *et al.* (V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." Biochem. Biophys. Res. Commun., 139: 424-430 [1986]), and McGee, *et al.*, (Z. A. McGee, *et al.*, "Local induction of tumor necrosis factor as molecular mechanism of mucosal damage by gonococci." Microbial Pathogenesis 12: 333-341 [1992]). Microtiter plates (96 well, Falcon, Microtest III) were inoculated with approximately 1 x 10^4 Vero cells (ATCC, CCL81) per well (100 µl) and incubated overnight at 37°C in the presence of 5% CO_2 to form Vero cell monolayers. rVT1 and rVT2 solutions were serially diluted in Medium 199 supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY), added to each well of the microtiter plates and incubated at 37°C for 18–24 hrs. Adherent (viable) cells were stained with 0.2% crystal

violet (Mallinckrodt) in 2% ethanol. Excess stain was rinsed away and the stained cells were solubilized by adding 100 µl of 1% SDS to each well. Absorbance of each well was measured at 570 nm. and the percent cytotoxicity of each test sample was calculated using the following formula:

% Vero Cytotoxicity = [1 - (Absorbance Sample/Absorbance Control)] x 100

To determine whether the rVT's possessed potency equivalent to published cytotoxicity values. a Vero cell cytotoxicity assay was performed (Figure 3). Between 0.01–10.000 pg of either rVT1 or rVT2 was added to Vero cells. The amounts of rVT causing 50% cell death (CD_{50}), as calculated by second degree polynomial curve fitting were 0.97 pg and 1.5 pg. for rVT1 and rVT2. respectively. These results are consistent with CD_{50} values reported previously for naturally occurring VT1 and VT2 in the range 1–35 pg and 1–25 pg. respectively (M. Petric *et al.*. Purification and biological properties of *Escherichia coli* verocytotoxin." FEMS Microbiol. Lett.. 41: 63-68 [1987]: V. L. Tesh. *et al.*. "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun.. 61: 3392-3402 [1993]: N. Dickie *et al.*. "Purification of an *Escherichia coli* Serogroup O157:H7 verotoxin and its detection in North American hemorrhagic colitis isolates." J. Clin. Microbiol.. 27: 1973-1978 [1989]; and U. Kongmuang. *et al.*. "A simple method for purification of Shiga or Shiga-Like toxin from *Shigella dysenteriae* and *Escherichia coli* O157:H7 by immunoaffinity chromatography." FEMS Microbiol. Lett.. 48: 379-383 [1987]). It has been observed that toxicity is lost with storage. explaining why higher amounts of toxin were used in the neutralization assays described below.

D. Mouse Lethal Dose Determination.

To verify rVT1 and rVT2 toxicity. male (20–22 g) CD-1 mice were injected intraperitoneally with varying amounts of rVT1 or rVT2 in 200 µL phosphate buffer. Doses were selected based on published LD_{50} values for VT1 and VT2 in CD-1 mice. To minimize the sacrifice of live animals. a full statistical toxin LD_{50} was not determined. Mice were observed for morbidity and mortality over 7-day period.

Further confirmation of rVT toxicity was obtained from mouse lethality experiments (Table 2). Mice were injected intraperitoneally with varying amounts of either rVT1 or rVT2 and observed 7 days for mortality. Within 72–120 hrs. post-injection. all of the mice died

from 100 ng of rVT1 or 10 ng of rVT2. respectively. This lethality study served as a verification of expected toxicity but not as a statistical determination of LD_{50}. Nonetheless. these results were consistent with toxicity studies which reported LD_{50} values in CD-1 mice of 0.4–2.0 µg for purified VT1 and 0.001–1.0 µg for purified VT2 (V. L. Tesh. *et al.*, "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun.. 61: 3392-3402 [1993]; and A. D. O'Brien. and G. D. LaVeck. "Purification and characterization of *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*," Infect. Immun. 40: 675-683 [1983]).

Table 2.
Lethality of rVT1 in CD-1 Mice

ng VT1 Injected	Survivors/Total	Hours Post-Injection
100	7/7	24 ± 2
	5/7	48 ± 2
	0/7	72 ± 2
10	7/7	24 ± 2
	7/7	48 ± 2
	7/7	72 ± 2
1.0	6/6	24 ± 2
	6/6	48 ± 2
	6/6	72 ± 2

Table 3.
Lethality of rVT2 in CD-1 Mice

ng VT2 Injected	Survivors/Total	Hours Post-Injection
10	3/6	48 ± 2
	2/6	72 ± 2
	0/6	120 ± 2
1.0	5/6	48 ± 2
	4/6	72 ± 2
	0/6	120 ± 2
0.1	6/6	48 ± 2
	6/6	72 ± 2
	6/6	120 ± 2

The recombinant toxins used in these studies thus appeared to contain protein components and toxicities consistent with literature reports for native toxins. Based on these structural and functional analyses, the rVT's were considered suitable as antigens to generate specific avian antibodies.

E. Antigen Preparation.

Lyophilized samples, rVT1 and rVT2 were received and each was reconstituted with 2.5 mL of deionized water to a final concentration of 100 μg/ml in phosphate buffer. To form a toxoid, the solutions were then treated with 0.4% glutaraldehyde (Mallinckrodt) at 4°C overnight and stored at -20°C thereafter. When needed, toxoid was thawed and mixed 5:1 (volume:volume) with GERBU adjuvant (C. C. Biotech Corporation, Poway, CA). White Leghorn laying hens were injected subcutaneously with 25 μg of either rVT1 or rVT2 toxoid in adjuvant at 2–3 week intervals.

EXAMPLE 2
PEG EXTRACTION OF EGG YOLK ANTIBODY

Hyperimmune eggs were collected after 3 immunizations with toxoid. Egg yolks were separated from whites, pooled according to their immunogen group and blended with 4 volumes of 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS). Polyethylene glycol

8000 (PEG) (Amresco. Solon, OH) was then added to a final concentration of 3.5% and the mixture centrifuged at 10,000 x g for 10 min. to remove the precipitated lipid fraction. IgY-rich supernatant was filtered through cheesecloth and PEG was again added to a final concentration of 12%. The solution was centrifuged as above and the resulting supernatant discarded. The IgY pellet was then dissolved in PBS to either the original (1X PEG IgY) or ¼ of the original (4X PEG IgY) yolk volume, filtered through a 0.45 μ membrane and stored at 4°C.

EXAMPLE 3
ANTITOXIN IMMUNOASSAYS

A. Enzyme Immunoassay (EIA).

EIA was used to monitor antibody responses during the immunization course. Wells of 96-well Pro-Bind microtiter plates (Falcon, through Scientific Products. McGaw Park, IL) were each coated with 1 μg of rVT's (not toxoid) in PBS overnight at 2–8°C. Wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound antigen, and the remaining protein binding sites were blocked with PBS containing 1 mg/ml BSA for 60 min. at room temperature (RT). IgY, diluted in PBS, was then added to the wells and incubated for 1 hr. at 37°C. Wells were washed as before to remove unbound primary antibody and incubated for 1 hr. at 37°C with alkaline phosphatase-conjugated rabbit-anti-chicken IgG (Sigma Chemical Company, St. Louis. MO) diluted 1:1000 in PBS-T. Wells were again washed and 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, MO) in 50 mM Na_2CO_3, 10 mM $MgCl_2$, pH 9.5 was added and allowed to incubate at RT. Phosphatase activity was detected by absorbance at 410 nm using a Dynatech MR700 microtiter plate reader.

Laying Leghorn hens were immunized as described above (Example 1, part E), using glutaraldehyde-treated rVT's. Following several immunizations, eggs were collected and IgY harvested by PEG fractionation. Figures 4 and 5 show rVT1 or rVT2 specific antibody responses detected using EIA at dilutions of the original yolk IgY concentration of 1:30,000 and 1:6,000, respectively. IgY fractionated similarly from unimmunized hens (*i.e.*, preimmune antibody) did not react with either antigen at test dilutions above 1:50. Although these EIA results indicate significant antibody responses, prior experience with other toxin antigens has shown that optimization of immunization regimens, including increasing the amount of

antigen. can yield titers in excess of 1:100.000 (B. S. Thalley, *et al.*."Development of an Avian Antitoxin to Type A Botulinum Neurotoxin." *in* Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects, B. R. DasGupta, (ed.) [Plenum Press. New York, 1993] pp. 467-472). As may be expected due to their structural homology and consistent with previous reports (*e.g.*, V. V. Padhye *et al.*. "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7." *J. Agr. Food Chem.*. 39: 141-145 [1989]; S. C. Head *et al.*. "Purification and characterization of verocytotoxin 2." FEMS Microbiol. Lett.. 51: 211-216 [1988]; and N. C. Strockbine *et al.*. "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*." Infect. Immun.. 50: 695-700 [1985]). Figures 4 and 5 also demonstrate that antibodies generated against one toxin cross-reacted *in vitro* with the other toxin.

B. Western Blot Analysis.

Western blots (Figure 6) performed to determine the reactivity of rVT antibodies against constituent VT polypeptides showed that rVT1 and rVT2 antibodies reacted with subunit A and fragment A1 of either toxin. and with subunit B and fragment A2 of rVT1 only. In this Figure. Panel A contains preimmune IgY. Panel B contains rVT1 IgY. and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 (2μg) and Lane 2 contains rVT2 (2 μg). Preimmune IgY was largely nonreactive to either rVT. Both rVT IgY preparations. however. failed to react with subunit B and fragment A2 of rVT2. Some explanations for this lack of measurable reactivity might include poor immunogenicity. denaturation of the immunogen during glutaraldehyde treatment. loss of conformational epitopes due to detergent or reducing agent. or poor transfer to nitrocellulose.

To resolve the high and low molecular weight components. 2 μg each of rVT1 and rVT2 were separated by SDS-PAGE (described above) and then transferred to nitrocellulose paper using the Milliblot-SDE system (Millipore. Medford. MA) according to the manufacturer's instructions. Paper strips were stained temporarily with Ponceau S (Sigma Chemical Company. St. Louis. MO) to visualize the polypeptides and then blocked overnight in PBS containing 5% dry milk. Each strip was agitated gently in IgY diluted in PBS-T for 2 hrs. at RT. Strips were each washed with three changes of PBS-T to remove unbound primary antibody and incubated for 2 hrs. at RT with goat anti-chicken alkaline phosphatase (Kirkegaard and Perry. Gaithersburg. MD) diluted 1:500 in PBS-T containing 1 mg/ml BSA. The blots were washed as before and rinsed in 50 mM Na_2CO_3. pH 9.5. Strips were

submerged in alkaline-phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Kirkegaard and Perry) until sufficient signal was observed. Color development was stopped by flooding the blots with water.

EXAMPLE 4

IN VITRO TOXIN NEUTRALIZATION: VERO CELL ASSAY

IgY neutralization of rVT1 and rVT2 was assessed using the modified Vero cytotoxicity assay described above (Example 1. part C). Various concentrations of IgY. diluted in Medium 199 supplemented with 5% fetal bovine serum (GIBCO). were mixed with sufficient toxin to cause 50% cell death and allowed to incubate at 37°C for 60 minutes. These toxin/antibody mixtures were then added to Vero cell-coated microtiter plate wells according to the procedure described above (Example 1. part C).

The toxin neutralization capacity of the rVT antibodies was analyzed first using a Vero cell toxicity assay. The results in Figure 7 show that rVT1 IgY neutralized completely the cytotoxic activity of rVT1 at an endpoint dilution of 1/320. Furthermore. rVT2 IgY neutralized the heterologous rVT1 toxin. but at a higher endpoint concentration.

In a similar experiment (see Figure 8), rVT1 and rVT2 antibodies were each able to neutralize rVT2 at equivalent endpoint dilutions. This strong cross-neutralization correlates with the observed strong cross-reactivity of VT1 IgY with VT2 A seen on Western blots (Figure 6). These results show that IgY antibodies are able to neutralize effectively VT cytotoxicity and that the antibodies can cross-neutralize structurally-related heterologous toxins.

EXAMPLE 5

TOXIN NEUTRALIZATION: MOUSE ASSAYS

A. Toxin Challenge Model.

IgY in PBS was premixed with a lethal dose of toxin (as determined above) and injected intraperitoneally into male CD-1 (20–22 gm) mice. Mice were observed for a 7-day period for signs of intoxication such as ruffled fur. huddling and disinclination to move. followed by hind leg paralysis. rapid breathing and death. Untreated. infected mice usually died within 12 hrs. after signs of severe illness (*i.e.*. within 48–72 hrs. post-injection).

Once it was demonstrated that rVT antibodies were able to neutralize rVT cytotoxicity *in vitro*, protection experiments were next performed in mice. First, animals were challenged with rVT premixed with rVT IgY to determine whether toxin lethality could be neutralized under conditions optimal for antigen/antibody reaction. Tables 4 and 5 show that antibodies premixed with the homologous toxin (*e.g.*, rVT1 with rVT1 IgY) prevented lethality of rVT. Preimmune IgY was unable to neutralize either toxin in these studies.

Table 4

Neutralization of rVT1 Using rVT IgY

100 ng rVT2 Premixed*	Survivors/Total	*p*
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

*Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Table 5

Neutralization of rVT2 Using rVT IgY

10 ng rVT1 Premixed*	Survivors/Total	*p*
Preimmune Antibody	0/12	
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

*Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Antibodies premixed with the heterologous toxin (*e.g.*, rVT2 with rVT1 IgY) also prevented lethality *in vivo*. These data are in contrast to previous observations where rabbit polyclonal antibodies generated against either toxin were cross-reactive with the heterologous toxin by EIA and Western blot, but were unable to neutralize the heterologous toxin in either Vero cell cytotoxicity and mouse lethality assays (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," FEMS Microbiol. Lett., 51: 211-216

[1988]; N. C. Strockbine *et al.*. "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from *Escherichia coli*." Infect. Immun., 50: 695-700 [1985]: and V. V. Padhye *et al.*, "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From *Escherichia coli* O157:H7." Biochem. Biophys. Res. Commun.. 139: 424-430 [1986]).

However, Head *et al.*. showed that VT2 B-subunit specific monoclonal antibodies neutralized VT1 weakly in a Vero cytotoxicity assay (S. C. Head. *et al.*. "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]). In a report by Donohue-Rolfe. *et al.*. a VT2 B subunit-specific monoclonal antibody neutralized both VT1 and VT2 completely in a Hela cytotoxicity assay (A. Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies." Infect. Immun., 57: 3888-3893 [1989]).

These results showed for the first time complete cross-neutralization in Vero cell cytotoxicity and mouse lethality assays. revealing that VT1 and VT2 do indeed share common neutralizing epitopes. These results may indicate that hens generate different antibody specificities as compared to mammals. and/or that differences in immunization methods might have maintained the immunogenicity of conformational epitopes necessary for cross-neutralization. Nonetheless. this cross-neutralization suggests that IgY antibodies may contain the range of reactivities essential for an effective antitoxin.

B. Viable organism infection model.

Streptomycin-resistant *E. coli* O157:H7 (strain 933 cu-rev) or *E. coli* O91:H21 (strain B2F1) (both kindly provided by Dr. Alison O'Brien. Dept. of Microbiology and Immunology. Uniformed Services University of the Health Sciences. Bethesda. MD) were used in a murine infection model described by Wadolkowski. *et al.* (E. A. Wadolkowski *et al.*. "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun.. 58: 2438-2445 [1990]). Organisms were grown in Luria broth and incubated overnight at 37°C in an Environ Shaker (Lab Line, Melrose Park. IL) (T. Maniatis *et al.*. <u>Molecular Cloning: a Laboratory Manual</u>, Cold Spring Harbor Laboratory. Cold Spring Harbor. N. Y., [1982]). Bacterial suspensions were centrifuged at 6700 x g for 5 minutes. The resulting pellet was then washed twice with sterile PBS and resuspended in sterile 20% (w/v) sucrose. Five to 8 week-old male CD-1 mice were provided drinking water containing 5 mg/ml streptomycin sulfate *ad libitum* for 24 hrs. Food and water were then withheld for

another 16–18 hrs. after which mice were challenged orally with 10^{10} streptomycin-resistant *E. coli* O157:H7 or O91:H21. Mice were housed individually and permitted food and water containing 5 mg/ml streptomycin sulfate. IgY was injected intraperitoneally at varying times post-infection and animals observed for both morbidity and mortality for 10 days.

To monitor bacterial colonization in animals. 1 gram of feces was collected. homogenized. and plated onto MacConkey agar medium (Difco Laboratories. Detroit. MI) containing 100 μg/ml streptomycin and incubated at 37°C as described by Wadolkowski. *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun.. 58: 2438-2445 [1990]). The serotype of *E. coli* O157:H7, 933 cu-rev excreted in feces was confirmed by slide agglutination with O– and H–specific antisera (Difco Laboratories. Detroit. MI).

Kidneys were removed from experimental animals and fixed in 10% buffered neutral formalin. Sections of parafilm-embedded tissue were stained with hematoxylin and eosin (General Medical Laboratories. Madison. WI) and examined by light microscopy. All tissue sections were coded to avoid bias before microscopic examination to determine renal pathology.

The toxin neutralization ability of rVT IgY was further studied using a streptomycin-treated CD-1 mouse infection model. This model was chosen because it produces definitive systemic pathology and reproducible mortality.

In contrast to previous studies by Wadolkowski. *et al.* (E. A. Wadolkowski *et al.*. "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]). where mice were given subunit-specific monoclonal antibodies *prior* to infection. the mice in this study were inoculated orally with 2×10^{10} viable *E. coli* O157:H7 (strain 933 cu-rev) and treated with rVT IgY 4 hrs. *following* inoculation. Fecal cultures showed that 10^7–10^8 challenge organisms per gram of feces were shed throughout the course of the experiment. thus confirming that infection was established. Tables 6 and 7 show that animals treated with either rVT1 or rVT2 IgY were protected from lethality caused by infection ($p<0.01$ and $p<0.001$. respectively) and that preimmune IgY failed to provide protection to the mice.

Table 6
Protection of Mice From *E. coli* O157:H7
With rVT1 IgY

IgY Treatment	Survivors/Total	*p*	Morbidity/Total
Preimmune Antibody	0/5		5/5
rVT1 Antibody	9/10	< 0.01	1/10

*IgY was administered intraperitoneally 4 hours following infection. and once daily for 10 days thereafter.

Table 7
Protection of Mice From *E. coli* O157:H7
With rVT2 IgY

IgY Treatment	Survivors/Total	*p*	Morbidity/Total
Preimmune Antibody	0/6		6/6
rVT2 Antibody	10/10	< 0.005	0/10

*IgY was administered intraperitoneally 4 hours following infection. and once daily for 10 days thereafter.

Renal histopathology (*see* Figure 9) of the control (preimmune IgY) animals showed dilation. degeneration and renal tubular necrosis with no glomerular damage. This is consistent with previous reports showing that renal tubular involvement occurs predominantly in this streptomycin-treated mouse infectivity model (E. A. Wadolkowski *et al.*. "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]). Importantly. none of the survivors exhibited similar signs of morbidity though treated with IgY 4 hrs. *after* infection (*see* Figure 9).

Furthermore. avian antibodies generated against rVT1 were able to prevent both mortality and morbidity in a mouse model where VT2 alone is implicated in the pathogenesis and lethality of *E. coli* O157:H7 strain 933 cu-rev (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun., 58: 3959-3965 [1990]).

To assess the broader utility of the IgY verotoxin antibodies in treating VTEC-associated disease. the mouse infectivity study was performed using a more virulent VTEC serotype known to produce VT2c—a structural variant of VT2—but not VT1 (S. W. Lindgren

et al., "Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model." Infect. Immun., 61: 3832-3842 [1993]).

Mice were inoculated orally with 5 x 10^9 *E. coli* O91:H21 (strain B2F1) and treated subsequently with IgY. Notably, the heterologous rVT1 IgY protected strongly against the lethal effects of the VT2c structural variant, even when administered as long as 10 hrs. following infection (Table 8). Ten hours was the longest treatment window tested in this study. Only 1 of the 8 animals treated with rVT1 IgY died ($p < 0.02$), and those that survived showed no overt signs of renal histopathology (*i.e.*, acute bilateral tubular necrosis). It can thus be concluded that rVT1 IgY completely neutralized toxicity of VT2c, indicating its potential as a therapeutic for at least one other pathogenic VTEC.

Table 8
Protection of Mice From *E. coli* O91:H21 With rVT1 IgY

IgY Treatment	Survivors/Total	*p*	Morbidity/Total
Preimmune Antibody	0/7		7/7
rVT1 Antibody	7/8	< 0.02	1/8

*IgY was administered intraperitoneally 10 hours following infection, and once daily for 8 days thereafter.

These Examples highlight several important findings supporting the feasibility of using verotoxin antitoxin. First, polyclonal IgY generated against either VT1 or VT2 from *E. coli* O157:H7, cross-reacted with and fully cross-neutralized the toxicity of the non-immunizing toxin both *in vitro* and *in vivo*. Second, recombinant toxins fully neutralized the toxicity of naturally-occurring toxins produced by *E. coli* O157:H7 during the course of infection. Third, antibodies generated against rVT1 from *E. coli* O157:H7 could prevent morbidity and mortality in mice infected orally with lethal doses of *E. coli* O91:H21, a particularly virulent strain which only produces VT2c, suggesting their utility in preventing systemic sequelae. Because VT1 is identical to Shiga-toxin (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]), VT antibodies may also be useful in preventing complications stemming from *Shigella dysenteriae* infection. Finally, animals treated with VT

IgY were protected against both death and kidney damage when treated as long as 10 hrs. after infection, supporting the hypothesis that a window for antitoxin intervention exists.

These studies strongly support the use of parenterally-administered. toxin-specific IgY as a antitoxin to prevent life-threatening complications associated with *E. coli* O157:H7 and other VTEC infections. It is contemplated that this approach would be most useful in preventing HUS and other complications when administered after the onset of bloody diarrhea and before the presentation of systemic disease.

The VT IgY developed in these studies were shown to react with and neutralize both recombinant and naturally-occurring VT. The antibody titers as measured by EIA are indicative of reasonable antibody production in the hen, however much higher production levels can be obtained with larger immunizing doses.

The results from these Examples clearly demonstrate the feasibility and provide the experimental basis for development of an avian antidote for *E. coli* O157:H7 verotoxins suitable for use in humans. In contrast to previous reports showing that rabbit polyclonal VT1 and VT2 antibodies cross-reacted, but did not cross-neutralize the heterologous toxin in Vero cytotoxicity or in mouse lethality studies (*e.g.*, V. V. Padhye *et al.*, "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from *Escherichia coli* O157:H7," J. Agr. Food Chem., 39: 141-145 [1989]; S. C. Head *et al.*, "Purification and characterization of verocytotoxin 2," FEMS Microbiol. Lett., 51: 211-216 [1988]; and N. C. Strockbine *et al.*, "Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*," Infect. Immun., 50: 695-700 [1985]), these data provide the first demonstration of cross-neutralization *in vivo*. Antibodies against one toxin neutralized completely the heterologous toxin in both Vero cytotoxicity and mouse lethality assays. Both rVT1 and rVT2 antibodies also prevented morbidity (as assessed by renal histopathology) and mortality in mice infected with lethal doses of *E. coli* O157:H7 – the etiologic agent in 90% of the documented cases of hemolytic uremic syndrome (HUS) in the U.S. (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1990]). With at least two other VTEC serotypes known to cause HUS, the finding that rVT1 antibodies neutralized a VT2 variant produced by *E. coli* O91:H21 suggests that avian polyclonal antibodies may provide an effective antidote against other verotoxin-producing *E. coli*. These data also show for the first time, that antibodies may be administered *after* infection and still protect against morbidity and mortality.

EXAMPLE 6
EXPRESSION OF TOXIN GENES

The previous Examples clearly showed that avian polyclonal antibodies to recombinant toxins protected animals infected with verotoxigenic *E. coli*. This Example includes expression of toxin genes (A and B subunits alone and together as whole toxins) in suitable prokaryotic expression systems to achieve high levels of VT antigen production.

The sequence of the toxin gene has been determined (*see e.g.*, M.P. Jackson *et al.*, "Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," 44:109 [1987]). The coding regions of the A and B subunits of VT-1 are listed in SEQ ID NOS:1 and 3, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-1 toxin are listed in SEQ ID NOS:2 and 4, respectively. The coding regions of the A and B subunits of VT-2 are listed in SEQ ID NOS:5 and 7, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-2 toxin are listed in SEQ ID NOS:6 and 8, respectively. In addition, SEQ ID NOS:9 and 10 list the sequences which direct the expression of a poly-cistronic RNA capable of directing the translation of both the A and B subunits from the VT-1 and VT-2 genes, respectively.

In choosing a strategy for recombinant VT antigen production, there are three primary technical factors to consider. First, the appropriate VT antigen components representing the spectrum of toxin epitopes encountered in nature must be utilized. Second, the protein antigens must be expressed at sufficient levels and purity to enable immunization and large-scale antibody purification. Third, the neutralizing epitopes must be preserved in the immunogen and immunoabsorbant. Approaches that offer the greatest promise for high level expression of periplasmically localized, native, affinity-tagged proteins were developed. Figure 10 shows the fusion constructs of VT components and affinity tags.

A. Expression of affinity-tagged C-terminal constructs.

The VT1 and VT2 A and B subunits (SEQ ID NOS:1, 3, 5 and 7) are cloned into the pET-23b vector (Novagen). This vector is designed to allow expression of native proteins containing C-terminal poly-His tags. The vector utilizes a strong T7 polymerase promoter to drive high level expression of target proteins. The methionine initiation codon is engineered to contain a unique *Nde*I restriction enzyme site (CATATG). The VT1 and VT2 genes are engineered to convert the signal sequence methionine codon into a *Nde*I site utilizing PCR

mutagenesis. PCR primers were designed which contain the sequence GCCAT fused to the first 20–24 bases of the genes (starting at the ATG start codon of the signal tag; SEQ ID NOS:12-19. see Table below). Upon PCR amplification, the 5' start codon of each gene is converted to an *Nde*I site, compatible with the pET-23 vector-encoded *Nde*I site, allowing cloning of the amplified genes into the vector without the addition of vector-encoded amino acids.

Primers containing the C-terminal 7 codons of each gene (21 bases) fused to the sequence CTCGAGCC were synthesized, in order to add a C-terminal poly-His tag to each gene. The underlined bases are an *Xho*I site, that is compatible with the *Xho*I site of the pET-23 vector. These primers precisely delete the native stop codons, and when cloned into the pET-23 vector, add a C-terminal extension of "LeuGluHisHisHisHisHisHis" (SEQ ID NO: 11). The following table lists the primer pairs are utilized to create PCR fragments containing the A and B subunits derived from VT-1 and VT-2 toxin genes suitable for insertion into the pET-23b vector.

Table 9

Primers

Toxin Gene and Subunit	N-terminal Primer	C-terminal Primer
VT-1 Subunit A	SEQ ID NO:12	SEQ ID NO:13
VT-1 Subunit B	SEQ ID NO:14	SEQ ID NO:15
VT-2 Subunit A	SEQ ID NO:16	SEQ ID NO:17
VT-2 Subunit B	SEQ ID NO:18	SEQ ID NO:19
VT-1 Subunits A and B	SEQ ID NO:12	SEQ ID NO:15
VT-2 Subunits A and B	SEQ ID NO:16	SEQ ID NO:19

Thus, utilizing PCR amplification with the above modified N- and C-terminal primers, the A and B subunits of VT1 and VT2 are expressed as proteins containing an 8 amino acid C-terminal extension bearing an poly-histidine affinity tag. The amino acid sequence of the histidine-tagged VT-1 A subunit produced by expression from the pET-23b vector is listed in SEQ ID NO:21 (the associated DNA sequence is listed in SEQ ID NO:20); the amino acid sequence of the histidine-tagged VT-1 B subunit is listed in SEQ ID NO:23 (the associated

DNA sequence is listed in SEQ ID NO:22); the amino acid sequence of the histidine-tagged VT-2 A subunit is listed in SEQ ID NO:25 (the associated DNA sequence is listed in SEQ ID NO:24); the amino acid sequence of the histidine-tagged VT-2 B subunit is listed in SEQ ID NO:27 (the associated DNA sequence is listed in SEQ ID NO:26).

Both subunits may be expressed from a single expression constructs by utilizing SEQ ID NOS:12 and 15 to prime synthesis of the VT-1 toxin gene and SEQ ID NOS:16 and 19 to prime synthesis of the VT-2 toxin gene. The resulting PCR products are cleaved with *Nde*I and *Xho*I, as described for the cloning of the subunit genes into the pET-23b vector. Expression of the A and B subunits from such an expression vector. results in the expression of a native A subunit and a his-tagged B subunit. As the A and B subunits assemble into a complex, the presence of the his-tag on only the B subunit is sufficient to allow purification of the holotoxin on metal chelate columns as described below.

The proofreading *Pfu* polymerase (Stratagene) is utilized for PCR amplification to reduce the error rate during amplification. Genomic DNA from an *E. coli* O157:H7 strain is utilized as template DNA. Following the PCR, the amplification products are digested with *Nde*I and *Xho*I and cloned into the pCR-Script SK cloning vehicle (Stratagene) to permit DNA sequence analysis of the amplified products. The DNA sequence analysis is performed to ensure that no base changes are introduced during amplification. Once the desired clones are identified by DNA sequencing, the inserts are then excised utilizing *Nde*I and *Xho*I, and cloned into a similarly cut pET-23b vector to create the expression constructs. According to the published sequences, neither the VT1 nor VT2 genes contain either of these restriction sites.

The poly-His-tagged proteins produced by expression of the VT-1 and VT-2 gene sequences in the pET-23b constructs are then purified by IMAC. This method uses metal-chelate affinity chromatography to purify native or denatured proteins which have histidine tails (*see e.g.*, K. J. Petty, "Metal-Chelate Affinity Chromatography," *in* Current Protocols in Molecular Biology, Supplement 24, Unit 10.11B [1993]).

B. Expression of Toxin Containing N-terminal Affinity Tags

Two expression systems, pMal-p2 and pFLAG-1 are utilized to attach an N-terminal affinity tag to the A subunits from the VT-1 and VT-2 toxins.

MBP-tagged constructs. To construct A chains containing the maltose binding protein (MBP) at the N-terminus of the A subunit, PCR amplified gene products are cloned into the

pMal-p2 vector (New England Biolabs) as C-terminal fusions to a periplasmically-secreted version of the MBP. The MBP selectively binds to amylose resins and serves as an affinity tag on the MBP/A subunit fusion protein. The pMal-p2 vector contains an engineered factor Xa cleavage site, which permits the removal of the affinity tag (*i.e.*, MBP) from the fusion protein after purification.

The MBP/A subunit fusions are generated as follows. The VT1 and VT2 A subunits are PCR-amplified utilizing the following DNA primers. SEQ ID NOS:28-31: SEQ ID NOS:28 and 29 comprise the 5' and 3' primers, respectively, for the amplification of the VT1 A subunit; SEQ ID NOS:30 and 31 comprise the 5' and 3' primers, respectively, for the amplification of the VT2 A subunit. In both cases, the 5' or N-terminal primer contains the sequence CG<u>GAATTC</u> fused to the first codon of the mature polypeptide (rather than the start of the signal peptide, since the MBP signal peptide is utilized). These 5' primers contain an engineered *Eco*RI site that is not contained internally in either gene, that is compatible with the *Eco*RI site of the pMal-p2 vector. The 3' or C-terminal primers incorporate an *Xho*I site as described above for the generation of the His-tagged toxins, but in this case, the 3' primer is designed to include the natural termination codon of the A subunits.

The genes are amplified, cloned into pCR-Script SK, and sequenced as described above. The inserts are then excised with *Eco*RI and *Xho*I, and cloned into *Eco*RI/*Sal*I-cleaved pMal-p2 vector (*Sal*I and *Xho*I sites are compatible). This construct allows expression and secretion of the VT1 and VT2 A subunit genes as C-terminal fusions with MBP. The amino acid sequence of the MBP/VT-1A fusion protein is listed in SEQ ID NO:33 (the associated DNA sequence is listed in SEQ ID NO:32). The amino acid sequence of the MBP/VT-2A fusion protein is listed in SEQ ID NO:35 (the associated DNA sequence is listed in SEQ ID NO:34).

The resulting fusion proteins are then affinity purified on an amylose column and the bound fusion protein is eluted under mild conditions by competition with maltose. The MBP N-terminal-tagged A subunits are cleaved with factor Xa and the MBP is removed by chromatography on an amylose column. The resulting A subunits which contain a 4 amino acid N-terminal extension are then used as immunogens.

Flag tag constructs. In an alternative embodiment, the VT1 and VT2 A subunit genes are engineered to contain the "flag tag" through the use of the pFLAG-1 vector system. The flag tag is located between the *Omp*A secretion signal sequence and the authentic N-

terminus of the target protein in the pFlag-1 vector. To construct N-terminal flag-tagged A chains, the *EcoRI/XhoI* A subunit PCR fragments (generated as described above for the MBP fusion proteins) are cloned into identically cleaved pFlag-1 vector (Eastman-Kodak), to produce an expression construct utilizing the *OmpA* signal peptide for secretion of A subunit fusion proteins containing the flag peptide at the N-terminus. After secretion, the periplasmic protein contains the N-terminal 8 amino acid flag tag, followed by 4 vector-encoded amino acids fused to the recombinant A subunit. The amino acid sequence of the flag tag/VT-1 A subunit fusion protein is listed in SEQ ID NO:37 (the associated DNA sequence is listed in SEQ ID NO:36). The amino acid sequence of the flag tag/VT-2 A subunit fusion protein is listed in SEQ ID NO:39 (the associated DNA sequence is listed in SEQ ID NO:38).

The flag tag fusion proteins are then purified by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (Antiflag M1; Eastman-Kodak). Mild elution of purified protein is achieved by chelating the calcium in the column buffer with ethylenediamine tetraacetic acid (EDTA).

C. Evaluation of fusion construct expression.

The fusion constructs described above are expressed in *E. coli* strain BL21, or T7 polymerase-containing derivatives [*e.g.*, BL21(DE3), BL21(DE3) pLysS, BL21(DE3)pLysE] (Novagen) for pET plasmids, and periplasmically-secreted recombinant protein purified by affinity chromatography. Recombinant proteins are analyzed for correct conformation by testing the following parameters:

a) It is believed that the B subunit must associate into pentamers to be conformationally correct. This is assessed by reducing and native SDS-PAGE analyses of native and chemically-cross-linked proteins and sizing HPLC;

b) It is believed that a properly folded A subunit is expected to retain its native enzymatic activity. This is tested by its capacity to inhibit protein synthesis in an *in vitro* toxicity assay;

c) It is believed that *in vitro* toxicity of assembled recombinant holotoxin is compared to commercially available holotoxins to determine whether recombinant A and B subunits can assemble into functional holotoxin. The

purified N-terminal-tagged A subunits (after cleavage and purification from MBP or untreated flag-tagged proteins) are combined *in vitro* with the corresponding B chains, and their toxicity evaluated utilizing a quantitative microtiter cytotoxicity assay, such as that described by M.K. Gentry and M. Dalrymple, "Quantitative Microtiter Cytotoxicity Assay for *Shigella* Toxin," J. Clin. Microbiol., 12:361-366 (1980).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: OPHIDIAN PHARMACEUTICALS, INC.

(ii) TITLE OF INVENTION: TREATMENT FOR VEROTOXIN-PRODUCING E. COLI

(iii) NUMBER OF SEQUENCES: 39

(iv) CORRESPONDENCE ADDRESS:
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(D) STATE: CALIFORNIA
(E) COUNTRY: UNITED STATES OF AMERICA
(F) ZIP: 94104

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: CARROLL, PETER G.
(B) REGISTRATION NUMBER: 32,837
(C) REFERENCE/DOCKET NUMBER: OPHD-02171

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 705-8410
(B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 945 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..945

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
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Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
        115                 120                 125

ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG      432
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225                 230                 235                 240

GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA      768
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
                245                 250                 255

AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC      816
Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
            260                 265                 270

AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA      864
Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
        275                 280                 285

GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG      912
Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 315 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 65                  70                  75                  80

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                 85                  90                  95

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Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
        195                 200                 205

Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
    210                 215                 220

Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
225                 230                 235                 240

Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
                245                 250                 255

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
            260                 265                 270

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
        275                 280                 285

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
    290                 295                 300

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser
305                 310                 315
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..267

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA      96
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
             20                  25                  30

AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA     144
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
         35                  40                  45

TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT CTC AGT GCG CAA     192
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
     50                  55                  60

ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA     240
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
 65                  70                  75                  80

GGG GGA TTC AGC GAA GTT ATT TTT CGT                                 267
Gly Gly Phe Ser Glu Val Ile Phe Arg
                 85
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
  1               5                  10                  15

Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
             20                  25                  30

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
         35                  40                  45

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
     50                  55                  60

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
 65                  70                  75                  80

Gly Gly Phe Ser Glu Val Ile Phe Arg
                 85
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 954 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..954

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT      48
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
  1               5                  10                  15

TCT TCG GTA TCC TAT TCC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA      96
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
             20                  25                  30

CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC     144
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
         35                  40                  45

CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC     192
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
     50                  55                  60

CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC     240
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
 65                  70                  75                  80

TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT     288
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
                 85                  90                  95

TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT     336
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
            100                 105                 110

TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC     384
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
        115                 120                 125

ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG     432
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
    130                 135                 140

GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT     480
Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145                 150                 155                 160

CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC     528
Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
                165                 170                 175

AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC     576
Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
            180                 185                 190

AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT     624
Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
        195                 200                 205

GTG TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG     672
Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
    210                 215                 220
```

CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA 720
Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225 230 235 240

GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG 768
Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
245 250 255

GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC 816
Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
260 265 270

GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT 864
Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
275 280 285

GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG 912
Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
290 295 300

TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA 954
Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys
305 310 315

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 318 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
1 5 10 15

Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
20 25 30

Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
35 40 45

Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
50 55 60

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
65 70 75 80

Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
85 90 95

Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
100 105 110

Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
115 120 125

Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
130 135 140

Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145 150 155 160

Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
165 170 175

Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
180 185 190

```
Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
        195                 200                 205

Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
    210                 215                 220

Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225                 230                 235                     240

Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
                245                 250                 255

Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
            260                 265                 270

Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
        275                 280                 285

Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
    290                 295                 300

Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..267

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT      48
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
  1               5                  10                  15

GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT      96
Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
             20                  25                  30

AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG     144
Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
         35                  40                  45

ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA     192
Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
     50                  55                  60

GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA     240
Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65                  70                  75                  80

TTT GCT GAA GTG CAG TTT AAT AAT GAC                                 267
Phe Ala Glu Val Gln Phe Asn Asn Asp
                 85
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
  1               5                  10                  15

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
             20                  25                  30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
         35                  40                  45

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
     50                  55                  60

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65                  70                  75                  80

Phe Ala Glu Val Gln Phe Asn Asn Asp
                 85
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1241 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATGAAAATAA TTATTTTTAG AGTGCTAACT TTTTTCTTTG TTATCTTTTC AGTTAATGTG      60

GTGGCGAAGG AATTTACCTT AGACTTCTCG ACTGCAAAGA CGTATGTAGA TTCGCTGAAT     120

GTCATTCGCT CTGCAATAGG TACTCCATTA CAGACTATTT CATCAGGAGG TACGTCTTTA     180

CTGATGATTG ATAGTGGCTC AGGGGATAAT TTGTTTGCAG TTGATGTCAG AGGGATAGAT     240

GCAGAGGAAG GGCGGTTTAA TAATCTACGG CTTATTGTTG AACGAAATAA TTTATATGTG     300

ACAGGATTTG TTAACAGGAC AAATAATGTT TTTTATCGCT TTGCTGATTT TTCACATGTT     360

ACCTTTCCAG GTACAACAGC GGTTACATTG TCTGGTGACA GTAGCTATAC CACGTTACAG     420

CGTGTTGCAG GGATCAGTCG TACGGGGATG CAGATAAATC GCCATTCGTT GACTACTTCT     480

TATCTGGATT TAATGTCGCA TAGTGGAACC TCACTGACGC AGTCTGTGGC AAGAGCGATG     540

TTACGGTTTG TTACTGTGAC AGCTGAAGCT TTACGTTTTC GGCAAATACA GAGGGGATTT     600

CGTACAACAC TGGATGATCT CAGTGGGCGT TCTTATGTAA TGACTGCTGA AGATGTTGAT     660

CTTACATTGA ACTGGGGAAG GTTGAGTAGC GTCCTGCCTG ACTATCATGG ACAAGACTCT     720

GTTCGTGTAG GAAGAATTTC TTTTGGAAGC ATTAATGCAA TTCTGGGAAG CGTGGCATTA     780

ATACTGAATT GTCATCATCA TGCATCGCGA GTTGCCAGAA TGGCATCTGA TGAGTTTCCT     840

TCTATGTGTC CGGCAGATGG AAGAGTCCGT GGGATTACGC ACAATAAAAT ATTGTGGGAT     900
```

TCATCCACTC TGGGGGCAAT TCTGATGCGC AGAACTATTA GCAGTTGAAC AGGGGGTAAA 960
TAAAGGAGTT AAGCATGAAA AAAACATTAT TAATAGCTGC ATCGCTTTCA TTTTTTTCAG 1020
CAAGTGCGCT GGCGACGCCT GATTGTGTAA CTGGAAAGGT GGAGTATACA AAATATAATG 1080
ATGACGATAC CTTTACAGTT AAAGTGGGTG ATAAAGAATT ATTTACCAAC AGATGGAATC 1140
TTCAGTCTCT TCTTCTCAGT GCGCAAATTA CGGGGATGAC TGTAACCATT AAAACTAATG 1200
CCTGTCATAA TGGAGGGGGA TTCAGCGAAG TTATTTTTCG T 1241

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1235 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGAAGTGTA TATTATTTAA ATGGGTACTG TGCCTGTTAC TGGGTTTTTC TTCGGTATCC 60
TATTCCCGGG AGTTTACGAT AGACTTTTCG ACCCAACAAA GTTATGTCTC TTCGTTAAAT 120
AGTATACGGA CAGAGATATC GACCCCTCTT GAACATATAT CTCAGGGGAC CACATCGGTG 180
TCTGTTATTA ACCACACCCA CGGCAGTTAT TTTGCTGTGG ATATACGAGG GCTTGATGTC 240
TATCAGGCGC GTTTTGACCA TCTTCGTCTG ATTATTGAGC AAAATAATTT ATATGTGGCA 300
GGGTTCGTTA ATACGGCAAC AAATACTTTC TACCGTTTTT CAGATTTTAC ACATATATCA 360
GTGCCCGGTG TGACAACGGT TTCCATGACA ACGGACAGCA GTTATACCAC TCTGCAACGT 420
GTCGCAGCGC TGGAACGTTC CGGAATGCAA ATCAGTCGTC ACTCACTGGT TTCATCATAT 480
CTGGCGTTAA TGGAGTTCAG TGGTAATACA ATGACCAGAG ATGCATCCAG AGCAGTTCTG 540
CGTTTTGTCA CTGTCACAGC AGAAGCCTTA CGCTTCAGGC AGATACAGAG AGAATTTCGT 600
CAGGCACTGT CTGAAACTGC TCCTGTGTAT ACGATGACGC CGGGAGACGT GGACCTCACT 660
CTGAACTGGG GGCGAATCAG CAATGTGCTT CCGGAGTATC GGGGAGAGGA TGGTGTCAGA 720
GTGGGGAGAA TATCCTTTAA TAATATATCA GCGATACTGG GGACTGTGGC CGTTATACTG 780
AATTGCCATC ATCAGGGGGC GCGTTCTGTT CGCGCCGTGA ATGAAGAGAG TCAACCAGAA 840
TGTCAGATAA CTGGCGACAG GCCTGTTATA AAAATAAACA ATACATTATG GGAAAGTAAT 900
ACAGCTGCAG CGTTTCTGAA CAGAAAGTCA CAGTTTTTAT ATACAACGGG TAAATAAAGG 960
AGTTAAGCAT GAAGAAGATG TTTATGGCGG TTTTATTTGC ATTAGCTTCT GTTAATGCAA 1020
TGGCGGCGGA TTGTGCTAAA GGTAAAATTG AGTTTTCCAA GTATAATGAG GATGACACAT 1080
TTACAGTGAA GGTTGACGGG AAAGAATACT GGACCAGTCG CTGGAATCTG CAACCGTTAC 1140
TGCAAAGTGC TCAGTTGACA GGAATGACTG TCACAATCAA ATCCAGTACC TGTGAATCAG 1200
GCTCCGGATT TGCTGAAGTG CAGTTTAATA ATGAC 1235

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Glu His His His His His His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCATATGAA AATAATTATT TTTAGAGTG 29

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCTCGAGAC TGCTAATAGT TCTGCGCAT 29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCATATGAA AAAAACATTA TTAATAGC 28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGCTCGAGAC GAAAAATAAC TTCGCTGAA 29

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCATATGAA GTGTATATTA TTTAAATGG 29

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCTCGAGTT TACCCGTTGT ATATAAAAAC 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCATATGAA GAAGATGTTT ATGGCG 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCTCGAGGT CATTATTAAA CTGCACTTC 29

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 969 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..969

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT 48

```
Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
 1               5                  10                  15

TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA       96
Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
            20                  25                  30

AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT      144
Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
        35                  40                  45

CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT      192
Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
    50                  55                  60

AGT GGC TCA GGG GAT AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT      240
Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
65                  70                  75                  80

GCA GAG GAA GGG CGG TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT      288
Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
                85                  90                  95

AAT TTA TAT GTG ACA GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT      336
Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
            100                 105                 110

CGC TTT GCT GAT TTT TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT      384
Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
        115                 120                 125

ACA TTG TCT GGT GAC AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG      432
Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
    130                 135                 140

ATC AGT CGT ACG GGG ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT      480
Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
145                 150                 155                 160

TAT CTG GAT TTA ATG TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG      528
Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
                165                 170                 175

GCA AGA GCG ATG TTA CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT      576
Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
            180                 185                 190

TTT CGG CAA ATA CAG AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT      624
Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
        195                 200                 205

GGG CGT TCT TAT GTA ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC      672
Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
    210                 215                 220

TGG GGA AGG TTG AGT AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT      720
Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
225                 230                 235                 240

GTT CGT GTA GGA AGA ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA      768
Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
                245                 250                 255

AGC GTG GCA TTA ATA CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC      816
Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
            260                 265                 270

AGA ATG GCA TCT GAT GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA      864
Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
        275                 280                 285
```

GTC CGT GGG ATT ACG CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG 912
Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
290 295 300

GGG GCA ATT CTG ATG CGC AGA ACT ATT AGC AGT CTC GAG CAC CAC CAC 960
Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His
305 310 315 320

CAC CAC CAC 969
His His His

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 323 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
1 5 10 15

Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
20 25 30

Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
35 40 45

Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
50 55 60

Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
65 70 75 80

Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
85 90 95

Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
100 105 110

Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val
115 120 125

Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly
130 135 140

Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser
145 150 155 160

Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
165 170 175

Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg
180 185 190

Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
195 200 205

Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn
210 215 220

Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser
225 230 235 240

Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
245 250 255

```
Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala
            260                 265                 270

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg
        275                 280                 285

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu
    290                 295                 300

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His
305                 310                 315                 320

His His His
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 294 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA      48
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
  1               5                  10                  15

AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA      96
Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
             20                  25                  30

AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA     144
Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
         35                  40                  45

TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTT CTC AGT GCG CAA     192
Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
     50                  55                  60

ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA     240
Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
 65                  70                  75                  80

GGG GGA TTC AGC GAA GTT ATT TTT CGT CTC GAG CAC CAC CAC CAC CAC     288
Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His
                 85                  90                  95

CAC TG                                                              294
His
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala
```

1 5 10 15

Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr
20 25 30

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu
35 40 45

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln
50 55 60

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly
65 70 75 80

Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His
85 90 95

His

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 981 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..981

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT 48
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
1 5 10 15

TCT TCG GTA TCC TAT TCC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA 96
Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
20 25 30

CAA AGT TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC 144
Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
35 40 45

CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC 192
Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
50 55 60

CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC 240
His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
65 70 75 80

TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT 288
Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
85 90 95

TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT 336
Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
100 105 110

TTT TCA GAT TTT ACA CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC 384
Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
115 120 125

ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG 432
Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
130 135 140

```
GAA CGT TCC GGA ATG CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT      480
Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145                 150                 155                 160

CTG GCG TTA ATG GAG TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC      528
Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
                165                 170                 175

AGA GCA GTT CTG CGT TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC      576
Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
            180                 185                 190

AGG CAG ATA CAG AGA GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT      624
Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
        195                 200                 205

GTG TAT ACG ATG ACG CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG      672
Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
    210                 215                 220

CGA ATC AGC AAT GTG CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA      720
Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225                 230                 235                 240

GTG GGG AGA ATA TCC TTT AAT AAT ATA TCA GCG ATA CTG GGG ACT GTG      768
Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
                245                 250                 255

GCC GTT ATA CTG AAT TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC      816
Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
            260                 265                 270

GTG AAT GAA GAG AGT CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT      864
Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
        275                 280                 285

GTT ATA AAA ATA AAC AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG      912
Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
    290                 295                 300

TTT CTG AAC AGA AAG TCA CAG TTT TTA TAT ACA ACG GGT AAA CTC GAG      960
Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu
305                 310                 315                 320

CAC CAC CAC CAC CAC CAC TG                                           981
His His His His His His
                325
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 326 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
  1               5                  10                  15

Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln
             20                  25                  30

Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
         35                  40                  45

Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn
     50                  55                  60
```

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val
65 70 75 80

Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn
85 90 95

Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg
100 105 110

Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser
115 120 125

Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu
130 135 140

Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr
145 150 155 160

Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser
165 170 175

Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe
180 185 190

Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro
195 200 205

Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly
210 215 220

Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg
225 230 235 240

Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val
245 250 255

Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala
260 265 270

Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro
275 280 285

Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala
290 295 300

Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu
305 310 315 320

His His His His His His
325

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 294 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..294

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT 48
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
1 5 10 15

```
GCA ATG GCG GCG GAT TGT GCT AAA GGT AAA ATT GAG TTT TCC AAG TAT      96
Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
             20                  25                  30

AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG     144
Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
         35                  40                  45

ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA     192
Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
     50                  55                  60

GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA     240
Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65                  70                  75                  80

TTT GCT GAA GTG CAG TTT AAT AAT GAC CTC GAG CAC CAC CAC CAC CAC     288
Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His
                 85                  90                  95

CAC TG                                                              294
His
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn
  1               5                  10                  15

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr
             20                  25                  30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp
         35                  40                  45

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr
     50                  55                  60

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly
 65                  70                  75                  80

Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His His
                 85                  90                  95

His
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
CGGAATTCAA GGAATTTACC TTAGACTTCT CG                                  32
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGCTCGAGTC AACTGCTAAT AGTTCTGC 28

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGGAATTCCG GGAGTTTACG ATAGACTTTT CG 32

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGCTCGAGTT ATTTACCCGT TGTATATAA 29

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2127

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG 48
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
1 5 10 15

ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA 96
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
20 25 30

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA 144
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
35 40 45

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG 192
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu

```
         50                  55                  60

CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC       240
His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
 65                  70                  75                  80

GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC       288
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                 85                  90                  95

GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG       336
Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG       384
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC       432
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
    130                 135                 140

AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG       480
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145                 150                 155                 160

CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC       528
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
                165                 170                 175

CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT       576
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
            180                 185                 190

TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC       624
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
        195                 200                 205

GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG       672
Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
    210                 215                 220

ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA       720
Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225                 230                 235                 240

GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG       768
Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
                245                 250                 255

GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA       816
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG       864
Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG       912
Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300
```

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TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT      960
Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                 320

AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG     1008
Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA     1056
Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
            340                 345                 350

GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC     1104
Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT     1152
Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT     1200
Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385                 390                 395                 400

AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC AAG     1248
Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys
                405                 410                 415

GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT TCG CTG     1296
Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu
            420                 425                 430

AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT TCA TCA     1344
Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser
        435                 440                 445

GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT AAT TTG     1392
Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu
    450                 455                 460

TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG TTT AAT     1440
Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn
465                 470                 475                 480

AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA GGA TTT     1488
Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe
                485                 490                 495

GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT TCA CAT     1536
Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His
            500                 505                 510

GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC AGT AGC     1584
Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser
        515                 520                 525

TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG ATG CAG     1632
Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln
    530                 535                 540

ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG TCG CAT     1680
Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His
545                 550                 555                 560

AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA CGG TTT     1728
Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe
                565                 570                 575

GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG AGG GGA     1776
Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly
```

```
            580                 585                 590

TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA ATG ACT        1824
Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr
        595                 600                 605

GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT AGC GTC        1872
Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val
    610                 615                 620

CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA ATT TCT        1920
Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser
625                 630                 635                 640

TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA CTG AAT        1968
Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn
                645                 650                 655

TGT CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT GAG TTT        2016
Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe
            660                 665                 670

CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG CAC AAT        2064
Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn
        675                 680                 685

AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG CGC AGA        2112
Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg
    690                 695                 700

ACT ATT AGC AGT TG                                                     2127
Thr Ile Ser Ser
705
```

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 708 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
  1               5                  10                  15

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
            20                  25                  30

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
        35                  40                  45

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
    50                  55                  60

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
 65                  70                  75                  80

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                85                  90                  95

Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
```

130 135 140

Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145 150 155 160

Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
165 170 175

Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
180 185 190

Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
195 200 205

Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
210 215 220

Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225 230 235 240

Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
245 250 255

Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
260 265 270

Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
275 280 285

Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
290 295 300

Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305 310 315 320

Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
325 330 335

Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
340 345 350

Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
355 360 365

Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
370 375 380

Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385 390 395 400

Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys
405 410 415

Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu
420 425 430

Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser
435 440 445

Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu
450 455 460

Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn
465 470 475 480

Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe
485 490 495

Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His
500 505 510

```
Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser
        515                 520                 525

Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln
    530                 535                 540

Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His
545                 550                 555                 560

Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe
                565                 570                 575

Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly
            580                 585                 590

Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr
        595                 600                 605

Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val
    610                 615                 620

Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser
625                 630                 635                 640

Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn
                645                 650                 655

Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe
            660                 665                 670

Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn
        675                 680                 685

Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg
    690                 695                 700

Thr Ile Ser Ser
705
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2136 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2136

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG      48
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
  1               5                  10                  15

ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA      96
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
             20                  25                  30

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA     144
Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
         35                  40                  45

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG     192
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
     50                  55                  60
```

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CAT CCG GAT AAA CTG GAA GAG AAA TTC CCA CAG GTT GCG GCA ACT GGC      240
His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
 65                 70                  75                  80

GAT GGC CCT GAC ATT ATC TTC TGG GCA CAC GAC CGC TTT GGT GGC TAC      288
Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                 85                  90                  95

GCT CAA TCT GGC CTG TTG GCT GAA ATC ACC CCG GAC AAA GCG TTC CAG      336
Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

GAC AAG CTG TAT CCG TTT ACC TGG GAT GCC GTA CGT TAC AAC GGC AAG      384
Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

CTG ATT GCT TAC CCG ATC GCT GTT GAA GCG TTA TCG CTG ATT TAT AAC      432
Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
    130                 135                 140

AAA GAT CTG CTG CCG AAC CCG CCA AAA ACC TGG GAA GAG ATC CCG GCG      480
Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145                 150                 155                 160

CTG GAT AAA GAA CTG AAA GCG AAA GGT AAG AGC GCG CTG ATG TTC AAC      528
Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
                165                 170                 175

CTG CAA GAA CCG TAC TTC ACC TGG CCG CTG ATT GCT GCT GAC GGG GGT      576
Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
            180                 185                 190

TAT GCG TTC AAG TAT GAA AAC GGC AAG TAC GAC ATT AAA GAC GTG GGC      624
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
        195                 200                 205

GTG GAT AAC GCT GGC GCG AAA GCG GGT CTG ACC TTC CTG GTT GAC CTG      672
Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
    210                 215                 220

ATT AAA AAC AAA CAC ATG AAT GCA GAC ACC GAT TAC TCC ATC GCA GAA      720
Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225                 230                 235                 240

GCT GCC TTT AAT AAA GGC GAA ACA GCG ATG ACC ATC AAC GGC CCG TGG      768
Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
                245                 250                 255

GCA TGG TCC AAC ATC GAC ACC AGC AAA GTG AAT TAT GGT GTA ACG GTA      816
Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

CTG CCG ACC TTC AAG GGT CAA CCA TCC AAA CCG TTC GTT GGC GTG CTG      864
Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

AGC GCA GGT ATT AAC GCC GCC AGT CCG AAC AAA GAG CTG GCG AAA GAG      912
Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300

TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT      960
Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                 320

AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG     1008
Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA     1056
Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
```

```
            340                        345                        350

GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC      1104
Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT      1152
Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAC AAT      1200
Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385                 390                 395                 400

AAC AAT AAC AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC CGG      1248
Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg
                405                 410                 415

GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT TCG TTA      1296
Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu
            420                 425                 430

AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA TCT CAG      1344
Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln
        435                 440                 445

GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT TAT TTT      1392
Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe
    450                 455                 460

GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT CAG GCG CGT TTT GAC CAT      1440
Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His
465                 470                 475                 480

CTT CGT CTG ATT ATT GAG CAA AAT AAT TTA TAT GTG GCA GGG TTC GTT      1488
Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val
                485                 490                 495

AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA CAT ATA      1536
Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile
            500                 505                 510

TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC AGT TAT      1584
Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr
        515                 520                 525

ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG CAA ATC      1632
Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile
    530                 535                 540

AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG TTC AGT      1680
Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser
545                 550                 555                 560

GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT TTT GTC      1728
Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val
                565                 570                 575

ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA GAA TTT      1776
Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe
            580                 585                 590

CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG CCG GGA      1824
Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly
        595                 600                 605

GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG CTT CCG      1872
Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro
    610                 615                 620
```

```
GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC TTT AAT      1920
Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn
625                 630                 635                 640

AAT ATA TCA GCG ATA CTG GGG ACT GTG GCC GTT ATA CTG AAT TGC CAT      1968
Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His
                645                 650                 655

CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT CAA CCA      2016
His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser Gln Pro
            660                 665                 670

GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC AAT ACA      2064
Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr
        675                 680                 685

TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG TCA CAG      2112
Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln
    690                 695                 700

TTT TTA TAT ACA ACG GGT AAA TA                                       2136
Phe Leu Tyr Thr Thr Gly Lys
705                 710
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 711 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
  1               5                  10                  15

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
             20                  25                  30

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
         35                  40                  45

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
     50                  55                  60

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
 65                  70                  75                  80

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr
                 85                  90                  95

Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
            100                 105                 110

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys
        115                 120                 125

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn
    130                 135                 140

Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala
145                 150                 155                 160

Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn
                165                 170                 175

Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly
            180                 185                 190
```

```
Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly
        195                 200                 205

Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu
    210                 215                 220

Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu
225                 230                 235                     240

Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp
                245                 250                 255

Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val
            260                 265                 270

Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu
        275                 280                 285

Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu
    290                 295                 300

Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn
305                 310                 315                     320

Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu
                325                 330                 335

Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys
            340                 345                 350

Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala
        355                 360                 365

Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp
    370                 375                 380

Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn
385                 390                 395                     400

Asn Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg
                405                 410                 415

Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu
            420                 425                 430

Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln
        435                 440                 445

Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe
    450                 455                 460

Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His
465                 470                 475                     480

Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val
                485                 490                 495

Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile
            500                 505                 510

Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr
        515                 520                 525

Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile
    530                 535                 540

Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser
545                 550                 555                     560

Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val
```

```
                565                     570                     575

Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Glu Phe
            580                     585                     590

Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr Pro Gly
        595                     600                     605

Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val Leu Pro
    610                     615                     620

Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn
625                     630                     635                     640

Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His
                645                     650                     655

His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser Gln Pro
            660                     665                     670

Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn Asn Thr
        675                     680                     685

Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys Ser Gln
    690                     695                     700

Phe Leu Tyr Thr Thr Gly Lys
705                     710
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 981 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..981

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT      48
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
  1               5                  10                  15

ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG AAG CTT GAA      96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
             20                  25                  30

TTC AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT     144
Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp
         35                  40                  45

TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT     192
Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile
     50                  55                  60

TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT     240
Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp
 65                  70                  75                  80

AAT TTG TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG     288
Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg
                 85                  90                  95

TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA     336
```

```
Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr
            100                 105                 110

GGA TTT GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT        384
Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe
        115                 120                 125

TCA CAT GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC        432
Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp
    130                 135                 140

AGT AGC TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG        480
Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly
145                 150                 155                 160

ATG CAG ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG        528
Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met
                165                 170                 175

TCG CAT AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA        576
Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu
            180                 185                 190

CGG TTT GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG        624
Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln
        195                 200                 205

AGG GGA TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA        672
Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val
    210                 215                 220

ATG ACT GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT        720
Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser
225                 230                 235                 240

AGC GTC CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA        768
Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg
                245                 250                 255

ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA        816
Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile
            260                 265                 270

CTG AAT TGT CAT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT        864
Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp
        275                 280                 285

GAG TTT CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG        912
Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr
    290                 295                 300

CAC AAT AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG        960
His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met
305                 310                 315                 320

CGC AGA ACT ATT AGC AGT TG                                             981
Arg Arg Thr Ile Ser Ser
                325
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 326 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
 1               5                  10                  15

Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
            20                  25                  30

Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp
        35                  40                  45

Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile
    50                  55                  60

Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp
65                  70                  75                      80

Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg
                85                  90                  95

Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr
            100                 105                 110

Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe
        115                 120                 125

Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp
    130                 135                 140

Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly
145                 150                 155                     160

Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met
                165                 170                 175

Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu
            180                 185                 190

Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln
        195                 200                 205

Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val
    210                 215                 220

Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser
225                 230                 235                     240

Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg
                245                 250                 255

Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile
            260                 265                 270

Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp
        275                 280                 285

Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr
    290                 295                 300

His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met
305                 310                 315                     320

Arg Arg Thr Ile Ser Ser
                325
```

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 990 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

```
ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT      48
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala
  1               5                  10                  15

ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC AAG AAG CTT GAA      96
Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp Lys Lys Leu Glu
             20                  25                  30

TTC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA CAA AGT TAT GTC TCT     144
Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser
         35                  40                  45

TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC CCT CTT GAA CAT ATA     192
Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile
     50                  55                  60

TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC CAC ACC CAC GGC AGT     240
Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser
 65                  70                  75                  80

TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC TAT CAG GCG CGT TTT     288
Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe
                 85                  90                  95

GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT TTA TAT GTG GCA GGG     336
Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly
            100                 105                 110

TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT TTT TCA GAT TTT ACA     384
Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr
        115                 120                 125

CAT ATA TCA GTG CCC GGT GTG ACA ACG GTT TCC ATG ACA ACG GAC AGC     432
His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser
    130                 135                 140

AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG GAA CGT TCC GGA ATG     480
Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met
145                 150                 155                 160

CAA ATC AGT CGT CAC TCA CTG GTT TCA TCA TAT CTG GCG TTA ATG GAG     528
Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu
                165                 170                 175

TTC AGT GGT AAT ACA ATG ACC AGA GAT GCA TCC AGA GCA GTT CTG CGT     576
Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg
            180                 185                 190

TTT GTC ACT GTC ACA GCA GAA GCC TTA CGC TTC AGG CAG ATA CAG AGA     624
Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg
        195                 200                 205

GAA TTT CGT CAG GCA CTG TCT GAA ACT GCT CCT GTG TAT ACG ATG ACG     672
Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr
    210                 215                 220

CCG GGA GAC GTG GAC CTC ACT CTG AAC TGG GGG CGA ATC AGC AAT GTG     720
Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val
225                 230                 235                 240

CTT CCG GAG TAT CGG GGA GAG GAT GGT GTC AGA GTG GGG AGA ATA TCC     768
Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser
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                245                 250                 255

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TGC CAT CAT CAG GGG GCG CGT TCT GTT CGC GCC GTG AAT GAA GAG AGT        864
Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser
        275                 280                 285

CAA CCA GAA TGT CAG ATA ACT GGC GAC AGG CCT GTT ATA AAA ATA AAC        912
Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn
    290                 295                 300

AAT ACA TTA TGG GAA AGT AAT ACA GCT GCA GCG TTT CTG AAC AGA AAG        960
Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys
305                 310                 315                 320

TCA CAG TTT TTA TAT ACA ACG GGT AAA TA                                 990
Ser Gln Phe Leu Tyr Thr Thr Gly Lys
                325                 330
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(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

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 1               5                  10                  15

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Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser
        35                  40                  45

Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile
    50                  55                  60

Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser
65                  70                  75                  80

Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe
                85                  90                  95

Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly
            100                 105                 110

Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr
        115                 120                 125

His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser
    130                 135                 140

Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met
145                 150                 155                 160

Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu
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Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg
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Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg
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        195                 200                 205

Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro Val Tyr Thr Met Thr
    210                 215                 220

Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Ile Ser Asn Val
225                 230                 235                 240

Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser
                245                 250                 255

Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn
            260                 265                 270

Cys His His Gln Gly Ala Arg Ser Val Arg Ala Val Asn Glu Glu Ser
        275                 280                 285

Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro Val Ile Lys Ile Asn
    290                 295                 300

Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala Phe Leu Asn Arg Lys
305                 310                 315                 320

Ser Gln Phe Leu Tyr Thr Thr Gly Lys
                325
```

CLAIMS

What is claimed is:

1. A method of treatment comprising:
   a) providing:
      i) antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable, and
      ii) an intoxicated subject; and
   b) administering said antitoxin to said subject.

2. The method of Claim 1 wherein said *Escherichia coli* verotoxin is recombinant.

3. The method of Claim 1 wherein said antitoxin is an avian antitoxin.

4. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.

5. The method of Claim 2 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

6. The method of Claim 1 wherein said subject is an adult.

7. The method of Claim 1 wherein said subject is a child.

8. The method of Claim 1 wherein said administering is parenteral.

9. The method of Claim 1 wherein said administering is oral.

10. A method of prophylactic treatment comprising:

a) providing:

i) an antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is parenterally administrable, and

ii) at least one subject is at risk of diarrheal disease; and

b) parenterally administering said antitoxin to said subject.

11. The method of Claim 10, wherein said subject is at risk of developing extra-intestinal complications of *Escherichia coli* infection.

12. The method of Claim 11, wherein said extra-intestinal complication is hemolytic uremic syndrome.

13. A composition comprising neutralizing antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amounts.

14. The composition of Claim 13 wherein said *Escherichia coli* verotoxin is a recombinant toxin.

15. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.

16. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

17. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

18. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1.

19. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2.

20. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.

21. The composition of Claim 14 wherein said antitoxin is an avian antitoxin.

22. A method of treatment of enteric bacterial infections comprising:

a) providing:

i) an avian antitoxin directed against at least one verotoxin produced by *Escherichia coli* in an aqueous solution in therapeutic amount that is parenterally administrable, and

ii) at least one infected subject; and

b) parenterally administering said avian antitoxin to said subject.

23. The method of Claim 18 wherein said *Escherichia coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7. O1:NM; O2:H5; O2:H7: O4:NM; O4:H10: O5:NM; O5:H16: O6:H1; O18:NM; O18:H7; O25:NM; O26:NM: O26:H11: O26:H32; O38:H21: O39:H4: O45:H2: O50:H7: O55:H7: O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM; O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10: O117:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM: O125:H8: O126:NM: O126:H8; O128:NM: O128:H2: O128:H8: O128:H12: O128:H25: O145:NM: O125:H25: O146:H21: O153:H25; O157:NM: O163:H19: O165:NM: O165:19: and O165:H25

24. The method of Claim 22 wherein said antitoxin comprises antitoxin directed against at least one *Escherichia coli* verotoxin.

25. The method of Claim 22 wherein said antitoxin is cross-reactive with at least one *Escherichia coli* verotoxin.

26. The method of Claim 22 wherein said antitoxin is reactive against toxins produced by members of the genus *Shigella*.

27. The method of Claim 26, wherein said antitoxin is reactive against toxins produced by *Shigella dysenteriae*.

28. A method for detecting *Escherichia coli* verotoxin in a sample comprising:

a) providing:

i) a sample;

ii) an antitoxin raised against *Escherichia coli* verotoxin; and

iii) a reporter reagent capable of binding said antitoxin; and

b) adding said antitoxin to said sample so that said antitoxin binds to the Escherichia coli verotoxin in said sample.

29. The method of Claim 28, wherein said antitoxin is an avian antitoxin.

30. The method of Claim 28, further comprising the steps of:

c) washing said unbound antitoxin from said sample;

d) adding said reporter reagent to said sample so that said reporter reagent binds to said bound antitoxin;

e) washing said unbound reporter reagent from said sample; and

f) detecting said reporter reagent bound to said antitoxin bound to the *Escherichia coli* verotoxin so that the verotoxin is detected.

31. The method of Claim 30 wherein said detecting is selected from the group consisting of enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay.

32. The method of Claim 30 wherein said sample is a biological sample.

33. The method of Claim 30 wherein said sample is an environmental sample.

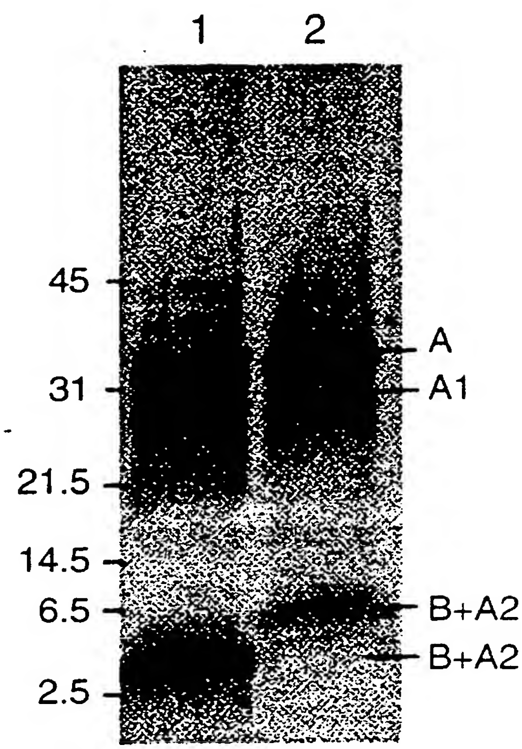
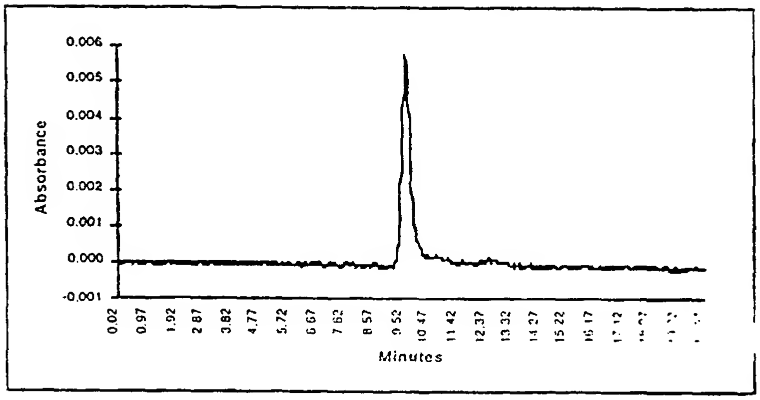


FIG. 1

Figure 2.

HPLC of rVT1



HPLC of rVT2

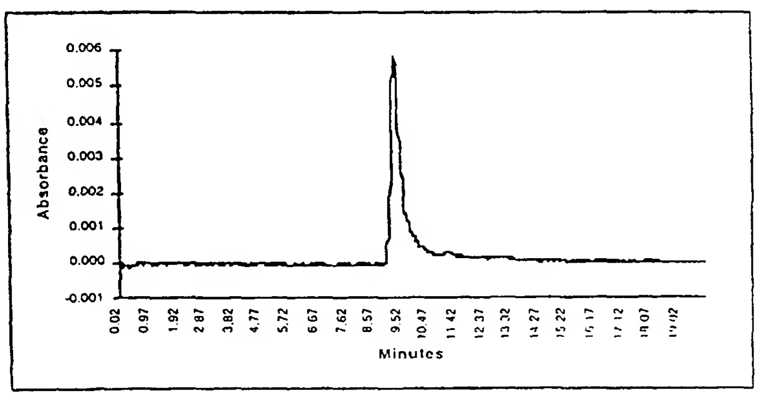


Figure 3.
rVT1 and rVT2 Toxicity in Vero Cell Culture

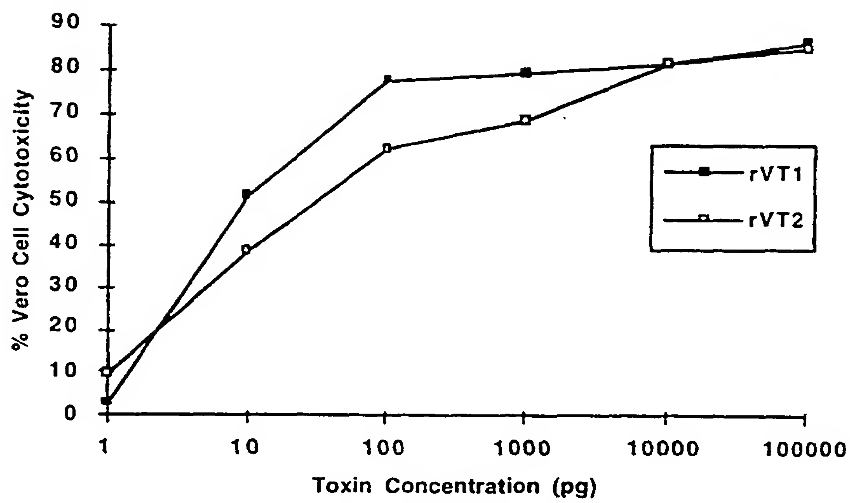


Figure 4.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT1

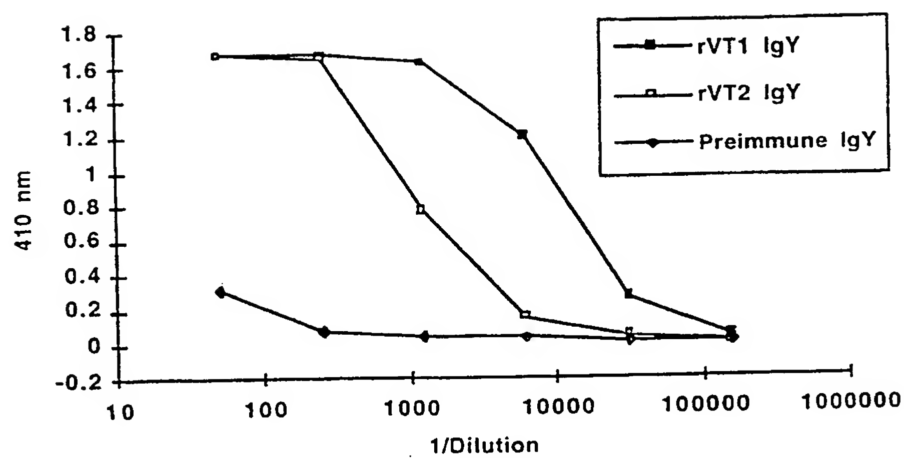
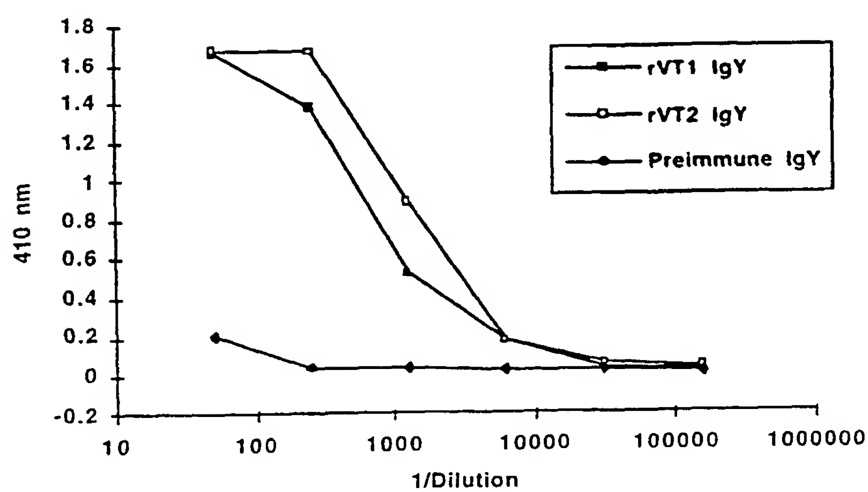


Figure 5.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT2



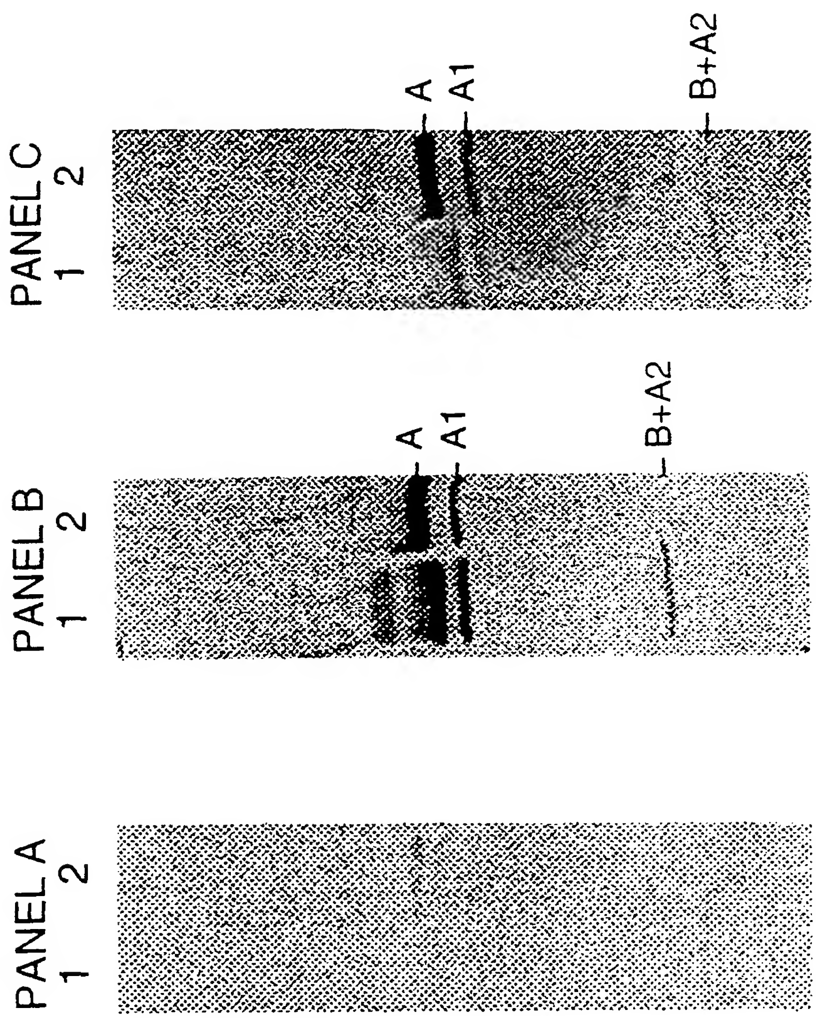


FIG. 6

Figure 7.

Neutralization of rVT1 Cytotoxicity in Vero Cells

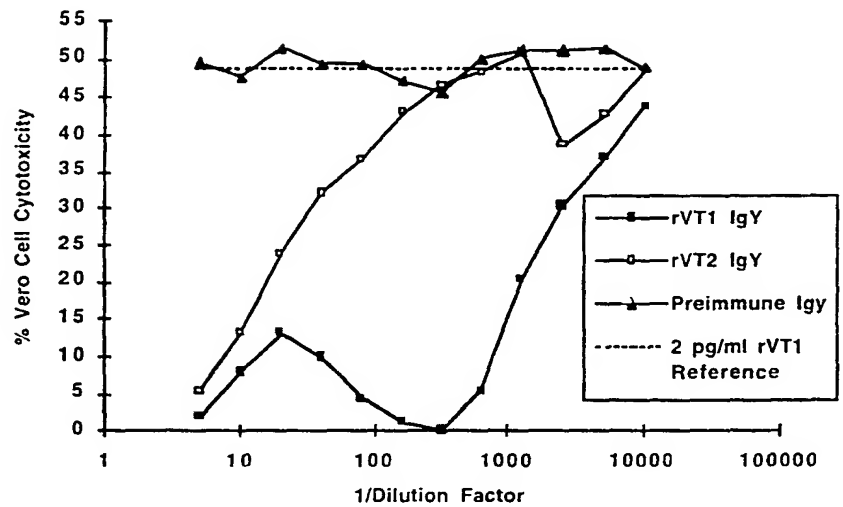
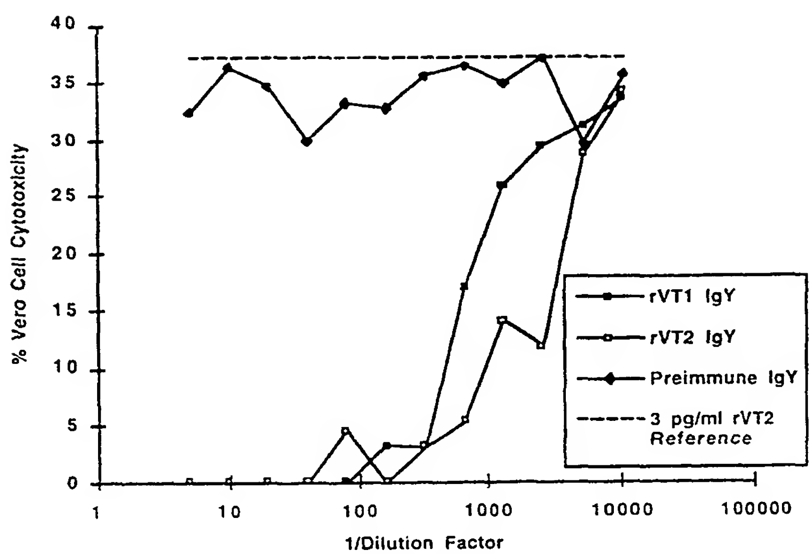


Figure 8.

Neutralization of rVT2 Cytotoxicity in Vero Cells



C

B

A

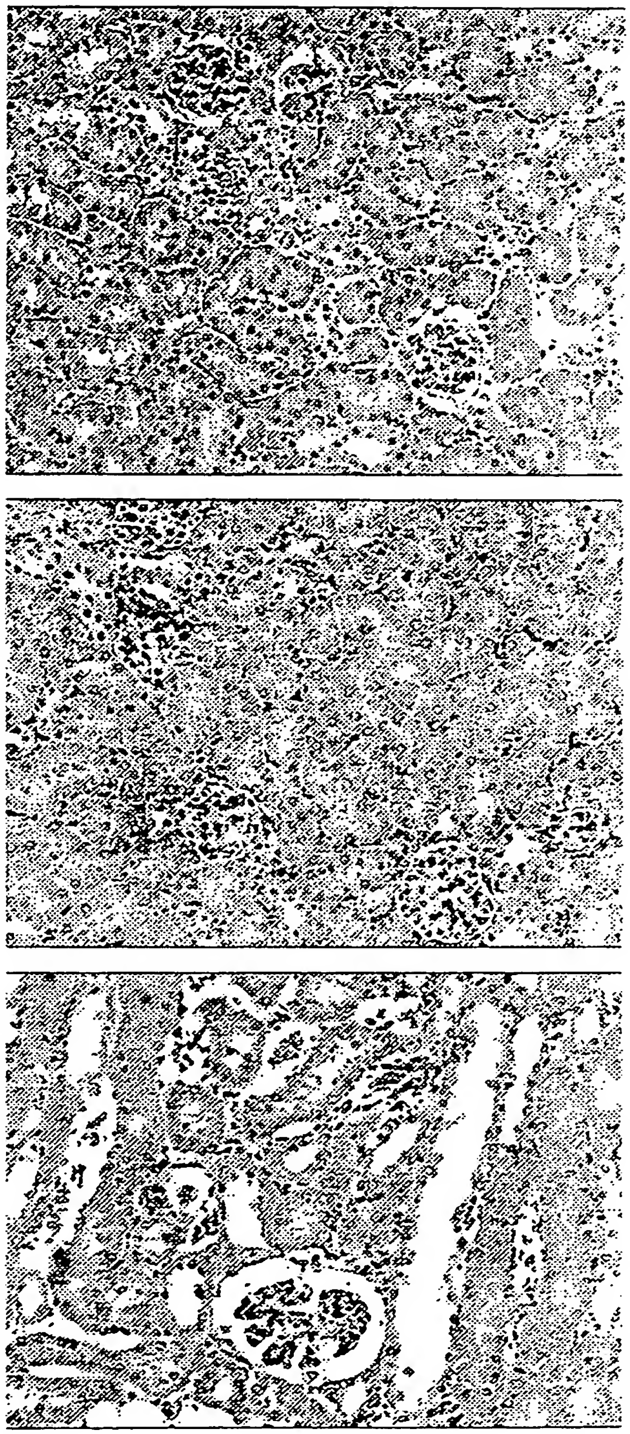


FIG. 9

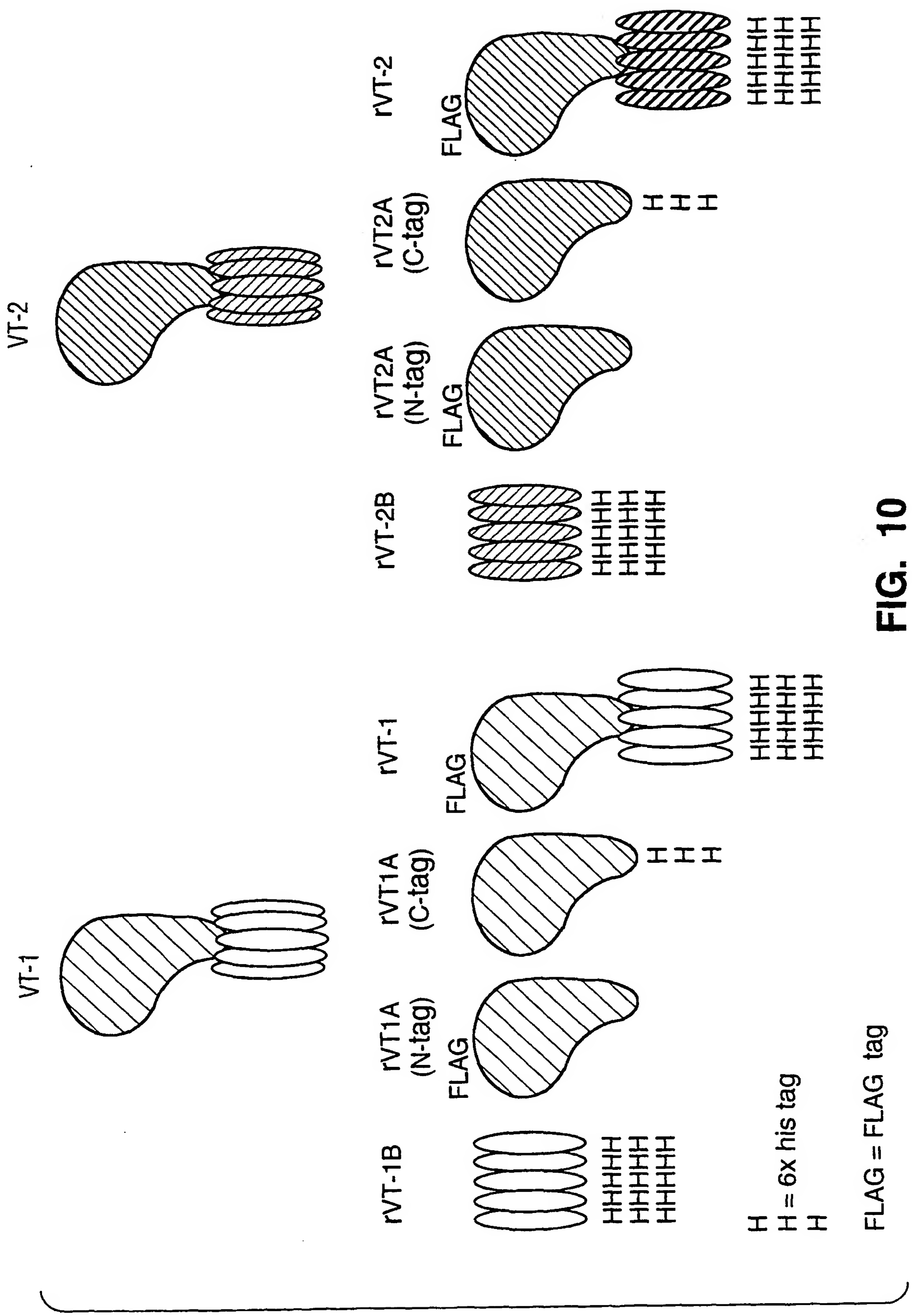

VT-1
VT-2
rVT-1B
rVT1A (N-tag)
FLAG
rVT1A (C-tag)
rVT-1
FLAG
HHHHH HHHHH HHHHH
H H H
rVT-2B
rVT2A (N-tag)
FLAG
rVT2A (C-tag)
rVT-2
FLAG
H = 6x his tag
FLAG = FLAG tag

FIG. 10

INTERNATIONAL S CH REPORT

Interna[redacted]application No.
PCT/US96/04093

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/00, 39/02; G01N 35/537
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOYD et al. Serological Responses to the B Subunit of Shiga-Like Toxin 1 and Its Peptide Fragments Indicate that the B Subunit Is a Vaccine Candidate To Counter the Action of the Toxin. Infection and Immunity. March 1991, Vol. 59, No. 3, pages 750-757.	1-33
Y	US 5,326,559 A (MILLER) 05 July 1994, columns 4-7.	1-33
X --- Y	US 5,164,298 A (LINGWOOD et al) 17 November 1992, columns 10-13.	28, 30, 31, 32, 33 ---------- 1-27 and 29
Y	US 4,748,018 A (STOLLE et al) 31 May 1988, column 4, lines 25-55.	3, 21, 22, 29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 JULY 1996	27 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RACHEL FREED Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,550,019 A (POLSON) 29 October 1985, column 4, lines 46-68.	3, 21, 22, 29
Y	US 5,204,097 A (ARNON et al) 20 April 1993, column 2, lines 1-16, column 3, lines 33-56 and column 5, lines 53-67.	2 and 14

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, MEDLINE, APS
search terms: verotoxin, verocytoxin, shiga, rvt1, rvt2, rslt1 or rslt2, vaccin? or treat?, recombinant